Biogeochemical Coupling and Microbial Regulation of Soil Carbon and Nitrogen Cycles in Grasslands

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A thesis
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Doctor of Philosophy

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by
Andrea Leptin

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"Land, then, is not merely soil; it is a fountain of energy flowing through a circuit of soils, plants, and animals."

Aldo Leopold, 'A Sand County Almanac'

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In New Zealand, a dramatic change to grasslands throughout the last two decades has been widespread conversion from dryland sheep farming to irrigated dairy farming. The detrimental impact of intensive dairy farming on groundwater quality requires new strategies to reduce carbon (C) and nitrogen (N) losses from grazed grasslands. Developing these strategies requires thorough understanding of the processes that regulate the coupling of the biogeochemical cycles of soil C and N. The objective of this work was to investigate the biogeochemical coupling of C and N cycles in grassland soils. To address this objective, two microcosm experiments in controlled conditions were undertaken and the findings were used to interpret observations made in a third experiment at a long-term field site. Carbon and nitrogen inputs to soil were manipulated in the microcosm experiments using different plant species and the addition of N, and long-term treatments at the field site consisted of mowing frequency and biomass retention or removal.

The objective of the first microcosm experiment was to determine the role of enhanced root-derived C availability on soil nitrification activity for five different grassland species: *Cichorium intybus* (chicory), *Lolium perenne* (perennial ryegrass), *Plantago lanceolata* (ribwort plantain), *Raphanus raphanistrum* (wild radish), and *R. sativus* (cultivated radish). These species were grown under controlled conditions for nine weeks and N was added at a low (no urea-N) or a high rate (550 kg urea-N ha^{-1}). Compared to the soils with low N addition, the high N addition rate resulted in an increase in water-extractable C concentrations and a decrease in potential nitrification activity. This suggests that increased C availability for microbial uptake may have stimulated microbial N immobilisation, resulting in reduced nitrification.

In the second microcosm study, the objective was to investigate the effects of increasing amounts of N addition on ecosystem C balance, C rhizodeposition, and the regulation of soil functional processes by changes in soil microbial community composition. *Lolium perenne* and *P. lanceolata* were grown for seven to eight weeks under controlled conditions and treated with increasing amounts of N (220, 300, 450, and 750 kg N ha^{-1}). A $^{13}CO_2$ pulse-labelling approach was used to trace photo-assimilated C through

the plant-soil-microbe system. Plant C and N uptake and C rhizodeposition increased with increasing N addition, with the greater amounts observed for *P. lanceolata* than those for *L. perenne*. There were also plant species-specific differences in the soil microbial community composition and microbial uptake of rhizodeposited C. Plant species-specific variation in microbial uptake of rhizodeposited C changed with increasing N addition, suggesting that microbial processing of rhizodeposited C from different plant species depends on N availability. Although the microbial community composition and the uptake of rhizodeposited C were closely related with soil respiration rates, there was no significant effect on soil N transformation rates. These findings suggest a decoupling of soil C and N cycles when N availability exceeds plant N uptake and highlights the important role of the soil microbial community composition in regulating soil C cycling.

Using an established long-term (>25 years) field experiment, the concept of ecological stoichiometry was used to link soil biogeochemical processes with microbial cycling of C, N, and P. The objective was to investigate relationships between microbial elemental limitation and soil organic matter concentrations, soil respiration and N mineralisation and nitrification rates. The long-term experiment is composed of 32 plots with eight different treatments: never mown, frequently and infrequently mown with clippings retained, and infrequently mown with clippings removed, all with and without N addition (50 kg N ha^{-1}). The C:N and C:P ratios were both greater for the soil microbial biomass than those ratios for the available soil substrates across all treatments, suggesting that the soil microbial community was primarily C-limited. The stoichiometric imbalance between available substrates and microbial elemental requirements were associated with changes in the soil microbial community composition and metabolic enzyme production. Significant relationships between the microbial community composition and SOM fractions, soil respiration rate, and C-acquiring enzyme activity highlighted the dependence of the soil microbial community on C. This may indicate that each microbial community has a specific C demand. The strong C limitation of the soil microbial community may explain the marginal effect of microbial and stoichiometric indices on soil N transformation rates.

This work has provided evidence that the composition and the stoichiometric elemental demand of the soil microbial community are key regulators for the biogeochemical processes that couple C and N cycles in grassland soils and that these processes can be influenced by grassland management practices. The findings demonstrate that root-derived soil C availability can be manipulated by supplying N for enhanced plant growth. Because the soil microbial community was shown to be primarily limited by C, increasing soil C availability could increase the stoichiometric N demand of the soil microbial community and this could lead to increases in microbial N immobilisation. Both, soil N status and plant species were shown to interactively affect the allocation of rhizodeposited C to different microbial groups. This may determine the fate of rhizodeposited C in the soil due to the critical role of soil microbial community composition in regulating soil C cycling. These findings can help with identifying and developing management practices that avoid uncontrolled decoupling of elemental cycles and C and N losses from grassland systems.

Keywords: Microbial communities, rhizodeposition, stable isotopes (^{13}C and ^{15}N), $^{13}CO_2$ pulse-labelling, ecosystem carbon balance, ecological stoichiometry, nitrification, nitrogen immobilisation, soil respiration, soil organic matter fractions

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Table of Contents

List of Tables

List of Figures

Chapter 1
Introduction

1.1 Context and scope

In New Zealand, about 55% of the total land area is occupied by grasslands (including unmanaged grasslands with shrubs) (Whitehead et al., 2018). These grasslands are crucially important for the nation's economy, as agricultural production represents the largest proportion of total export revenue (77%) and about half of this is derived from the dairy industry (Journeaux et al., 2017; Whitehead et al., 2018). Agricultural use of grasslands has shifted over recent decades and irrigated dairy farming has superseded dryland sheep farming (MacLeod and Moller, 2006). Between 1994 and 2017, the number of dairy cattle has increased by 70%, while the number of sheep has decreased by 44% (Ministry for the Environment and Stats NZ, 2019). Similarly, the area of land that is under irrigation was 94% higher in 2017 than it was in 2002 (Ministry for the Environment and Stats NZ, 2019). While this dramatic increase in grassland intensification has been beneficial for export revenue, it has been detrimental to the environment, including groundwater quality, due to, *inter alia*, high losses of soil carbon (C) and nitrogen (N) (Cameron et al., 2013; Foote et al., 2015; McDowell et al., 2011; Parfitt et al., 2006; Schipper et al., 2007; Whitehead et al., 2018). The environmental impact of dairy farming requires strategies to improve sustainability, including the reduction of C and N losses (Environment Canterbury, 2018; Jay, 2007). However, identifying these strategies remains challenging due to limited understanding of the biotic mechanisms that determine C and N retention (Conant et al., 2017; de Vries and Bardgett, 2012; Schipper et al., 2007; Whitehead et al., 2018).

The mobility and leaching susceptibility of soil N depends mostly on the concentration of nitrate (NO_3^-), which is primarily produced by microbial nitrifiers through nitrification (Cameron et al., 2013; Canfield et al., 2010). Nitrification increases when the availability of ammonium (NH_4^+) or ammonia (NH_3) exceeds plant N uptake, which occurs regularly in intensive grasslands under deposited urine patches, increasing the risk of NO_3^- leaching (Crews and Peoples, 2005; Robertson and Groffman, 2015; Selbie et al., 2015). To reduce the risk of NO_3^- leaching losses, a better balance between N availability and plant N demand is needed (Robertson and Vitousek, 2009). A potential way to achieve this balance is by increasing immobilisation of excess N by heterotrophic microorganisms, where subsequent N mineralisation can regulate the supply of available N for plant uptake (Robertson and Vitousek, 2009). Because heterotrophic microorganisms require C and N in relatively constrained proportions, their stoichiometric N demand increases with increasing C uptake (Booth et al., 2005; Cleveland and Liptzin, 2007; Sterner and Elser, 2002). Research investigating the impact of increased C supply on microbial N immobilisation is needed, since previous studies are scarce and the findings of the effects of an increased supply of C on N immobilisation are not consistent (Fisk et al., 2015; Zhao et al., 2018).

Carbon compounds available for microbial uptake, such as carbohydrates, often account for the largest proportion of root exudates released by living plant roots as rhizodeposits (Gunina and Kuzyakov, 2015; Hütsch et al., 2002; Jones et al., 2004). Rhizodeposition can constitute approximately 20 to 60% of photosynthetically assimilated C (Neumann and Römheld, 2012), and the amount and composition of rhizodeposits depends on the plant species, their physiological stage, and environmental conditions, such as soil N availability (Badri and Vivanco, 2009; Bais et al., 2006; Jones et al., 2009, 2004; Nguyen, 2003). Although increased soil N availability has been associated with an increase in C rhizodeposition and stabilisation in soil organic C (SOC) (Bowsher et al., 2018; Carvalhais et al., 2011; Xu et al., 2021), the number of observations are limited and reported results are often inconsistent between sites and treatments (Bradford et al., 2008; Khan et al., 2007; Liu and Greaver, 2010; Riggs et al., 2015; Ye et al., 2018). Currently, there is limited knowledge of the mechanisms that determine how N availability affects C rhizodeposition and SOC concentrations (Bowsher et al., 2018; Bradford et al., 2008; Khan et al., 2007; Liu and Greaver, 2010).

It is clear that the C and N cycles in grassland soils are closely coupled, so that changes in the cycling in one element will inevitably affect biogeochemical processes involved in the cycling of the other element (Reay et al., 2008; Rumpel et al., 2015; Soussana and Lemaire, 2014). To ensure that grassland management practices do not promote decoupled C and N cycles and, as a result, to C and N losses, an enhanced understanding of the interactions between the C and N cycles and the mechanisms that regulate the coupling of these cycles is required (Rumpel and Chabbi, 2019).

Soil C and N cycles have often been studied separately because the mechanisms that couple C to N are insufficiently understood and difficult to investigate experimentally. This thesis aimed to fill this knowledge gap by linking changes in biogeochemical pools of soil C and N with microbially regulated processes using laboratory and field experiments.

1.2 Thesis aims and objectives

The main objective of this PhD research work was to investigate the biogeochemical coupling of soil C and N cycles in grassland systems with the overall aim to inform the use of management practices to reduce C and N losses from grazed grasslands. The following specific objectives were developed to address the main objective of this thesis:

1. Identify the effects of increased available C supply from plant roots on the regulation of soil nitrification for different plant species (Chapter 3).

2. Determine the effects of increasing N availability on ecosystem C balance, C rhizodeposition, and regulation of C and N cycling by the microbial community under two grassland plant species (Chapter 4).

3. Determine the relationships between microbial biomass stoichiometry, available substrate stoichiometry, and the microbial community composition under different grassland management practices (Chapter 5).

1.3 Thesis outline

This thesis comprises 5 Chapters, with experimental Chapters 3 to 5 prepared in a manuscript format for submission to peer-reviewed journals. Therefore, the experimental chapters each contain a 'Materials and Methods' section. To avoid repetition, two manuscripts prepared for publication in peer-reviewed journals were combined into one thesis chapter (Chapter 4), because the results were obtained from a single experiment.

Chapter 1 introduces the thesis topic and outlines the aims and objectives.

Chapter 2 provides a basis for the thesis by reviewing and summarising literature on the general thesis themes of C and N cycling in grassland systems, the coupling of these cycles, and the concept of ecological stoichiometry in plant-soil-microbe interactions.

Chapter 3 presents experimental results from a controlled-environment study using microcosms to determine the response of potential nitrification activity to increased root-derived available C, which was stimulated by high N availability.

Chapter 4 presents experimental data from a $^{13}CO_2$ pulse-labelling experiment using microcosms to trace C through the plant-soil-microbe system in relation to the effects of N availabilities on C rhizodeposition and its utilisation by soil microorganisms.

Chapter 5 presents experimental results from an established long-term field trial (>25 years) where the relationships between microbial elemental demand, substrate stoichiometry, and microbial community composition and function were investigated.

Chapter 6 synthesises the main findings of the experimental studies, provides concluding remarks, and suggestions for future research.

Chapter 2
Literature review

2.1 Temperate grassland ecosystems

Grasslands are defined as ecosystems primarily consisting of herbaceous species with a paucity of trees and shrubs (Wilsey, 2018). In 2018, grasslands covered about 24.8% of the Earth's terrestrial surface area and about 67.4% of the world's agricultural land area (FAO, 2018). Because of their widespread coverage, grasslands are crucial for the provision of ecosystem services, such as maintaining high soil organic matter (SOM) stocks that are known to enhance soil and water quality by retaining C and nutrients (Duru et al., 2019; Lal, 2004; Lehmann and Kleber, 2015). Further pressure to increase grassland productivity exposes these ecosystem services to risk, because intensive use of grasslands for livestock grazing can lead to SOM decomposition and losses of soil C and nutrients, for example N (Cameron et al., 2013; Conant et al., 2017; Fornara et al., 2016; McSherry and Ritchie, 2013; Sanderman et al., 2017; Smith et al., 2016). However, the development of sustainable grassland management strategies is constrained by poor understanding of how different grassland management practices affect grassland soil C and N retention (de Vries and Bardgett, 2016, 2012; McSherry and Ritchie, 2013; Weitzman and Kaye, 2016).

2.2 Nitrogen cycling in grassland ecosystems

Nitrogen is an essential nutrient for growth, maintenance, and reproduction of all organisms (Galloway, 1998; Vertès et al., 2019). The N concentration in soils typically ranges between 0.1 and 0.6% for different soil types, which corresponds to about 2 to 12 Mg N ha^{-1} (Cameron et al., 2013). Nonetheless, primary productivity in temperate grasslands is often limited by N (LeBauer and Treseder, 2008).

Nitrogen exists in a wide variety of organic and inorganic forms, with four of them representing the major species in soils (Cameron et al., 2013): (1) SOM, (2) living soil macro- and microorganisms, (3) NH_4^+ bound to reactive mineral surfaces and organic matter, and (4) inorganic N forms in soil solution, such as NH_4^+, NO_3^-, and nitrite (NO_2^-). While N is involved in many biogeochemical transformations when it cycles through the terrestrial system (Figure 2.1), this review will focus on the following processes that are carried out by soil microorganisms: nitrification, microbial N mineralisation and immobilisation.

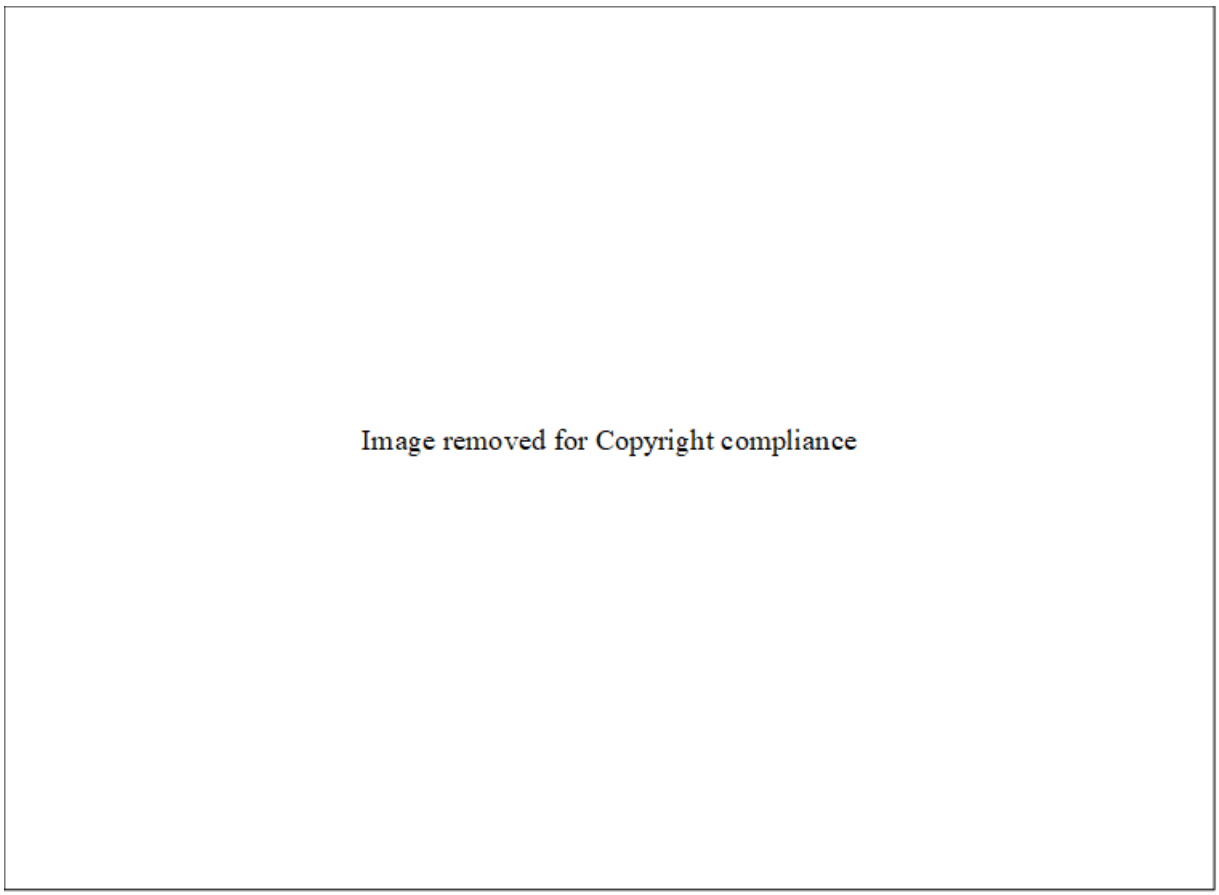

Figure 2.1. Schematic representation of the terrestrial nitrogen cycle (from Cameron et al., 2013).

2.2.1 Nitrification

Nitrification is the oxidative conversion of ammonia (NH_3) and NH_4^+ to NO_3^- via hydroxylamine (NH_2OH), nitric oxide (NO) and NO_2^- (Sayavedra-Soto and Arp, 2011; Stein, 2019). The first and rate-limiting step of the nitrification process, the oxidation of NH_3 to NO_2^-, is mainly controlled by chemolithotrophic ammonia-oxidising archaea (AOA) and bacteria (AOB) using their membrane-bound ammonia monooxygenase enzymes (Kowalchuk and Stephen, 2001; Prosser and Nicol, 2012; Stein and Klotz, 2016). Ammonia oxidation is a catabolic process and represents the primary energy source for AOA and AOB (Figure 2.2) (Prosser and Nicol, 2012; Sayavedra-Soto and Arp, 2011). While AOA abundance often exceeds that of AOB and can play an important role for nitrification in acidic and N-poor soils (Gubry-Rangin et al., 2010; Lu and Jia, 2013; Prosser and Nicol, 2012), AOB typically contribute more to nitrification in N-rich environments, such as grazed grasslands, because their N uptake capacity saturates at a higher concentration than that of AOA (Carey et al., 2016; Di et al., 2009; Jia and Conrad, 2009).

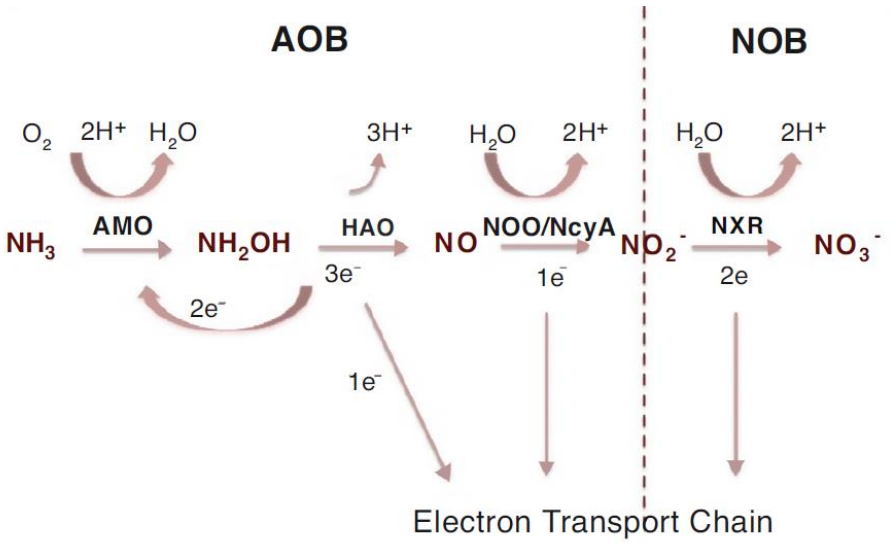

Figure 2.2. Pathways for energy acquisition by ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) and involved enzymes. AMO = ammonia monooxygenase, HAO = hydroxylamine dehydrogenase, NOO/NcyA = nitric oxide oxidoreductase/nitrosocyanin, NXR = nitrite oxidoreductase (reprinted from Stein, 2019, Copyright (2019), with permission from Elsevier).

The nitrification process is of particular interest in grazed grassland systems because high N loadings from urine deposited by grazing livestock increases nitrification rates, leading to high concentrations of mobile NO_3^- (Canfield et al., 2010; Coskun et al., 2017a; Prosser, 2011; Selbie et al., 2015). In contrast to NH_4^+, which many soils can retain by electrostatic sorption to soil minerals, NO_3^- is poorly retained and it is highly susceptible to leaching (Canfield et al., 2010; Coskun et al., 2017a). Nitrogen leaching losses from grazed systems in New Zealand typically range between about 2 to 200 kg N ha^{-1} $year^{-1}$ with approximately 70 to 90% of total N leached derived from grazing livestock (Ledgard et al., 2009; Monaghan et al., 2007). Nitrogen leaching losses increase exponentially with increasing N loading rates (Figure 2.3) (Ledgard et al., 2009) and the dramatic increase in grassland intensification in New Zealand has been accompanied by a substantial increase in NO_3^- leaching losses from grazing livestock. These losses have been estimated to exceed 50 kg N ha^{-1} in areas with high livestock production (Figure 2.4) (Ministry for the Environment and Stats NZ, 2019).

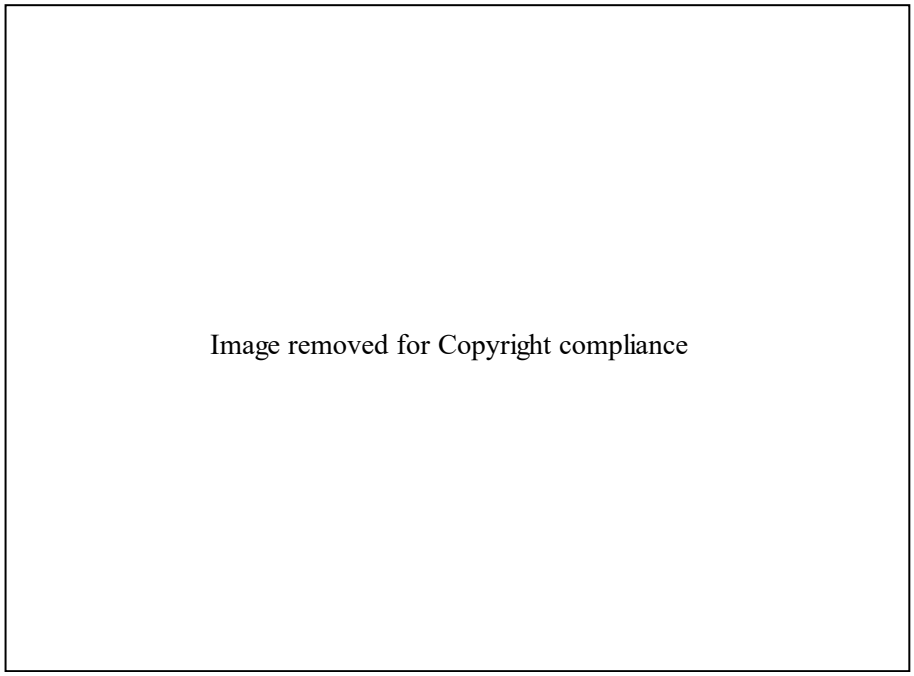

Figure 2.3. Effect of total nitrogen (N) inputs on nitrate-N leached from grazed grassland systems with different plant species composition. Data are derived from studies in New Zealand, France, the United Kingdom, and Denmark (from Ledgard et al., 2009).

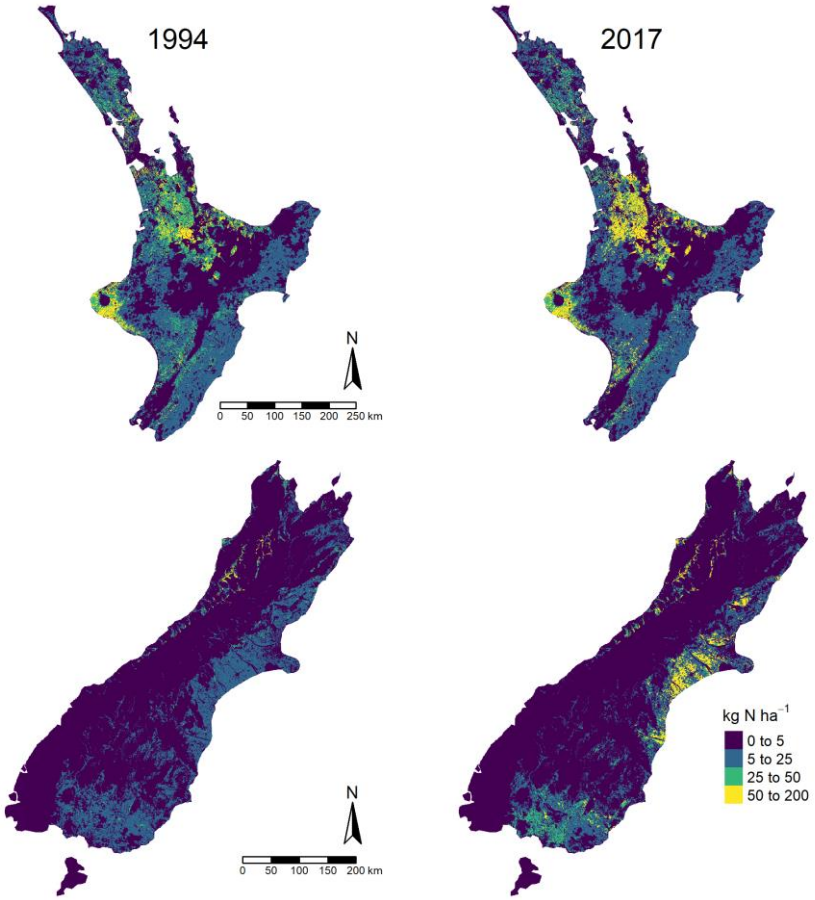

Figure 2.4. Modelled nitrate-nitrogen (in kg N ha^{-1}) leached from livestock for North Island (upper) and South Island (lower) New Zealand for 1994 and 2017. Data by courtesy of Anne-Gaelle Ausseil, Manaaki Whenua – Landcare Research, New Zealand.

Nitrogen leaching losses represent not only a loss of soil fertility but also an increased risk for N pollution of water bodies (Coskun et al., 2017a; Fowler et al., 2013; Galloway et al., 2008). Therefore, inhibiting nitrification and thereby the production of NO_3^- can reduce environmental risks (Robertson and Groffman, 2015). Among several approaches to inhibit nitrification (Cai and Akiyama, 2017; Coskun et al., 2017a; Di and Cameron, 2016; Qiao et al., 2015), it has been postulated that increasing N immobilisation by soil microorganisms can reduce the amount of N that is available for nitrification and the risk for N loss during times of low plant N uptake (Crews and Peoples, 2005; Robertson and Vitousek, 2009).

2.2.2 Nitrogen mineralisation and immobilisation

Nitrogen is mineralised when it is converted from an organic to an inorganic form, for example, through microbial SOM decomposition that releases NH_4^+ (Jansson and Persson, 1982; Li et al., 2019). Because N mineralisation produces N that is available for nitrification, N mineralisation rates are typically closely associated with nitrification rates (Booth et al., 2005; Cameron et al., 2013; Colman and Schimel, 2013).

Nitrogen immobilisation is the inverse process of mineralisation, referring to the conversion of inorganic N species to organic N species, for example through uptake and fixation of NH_4^+ and NO_3^- by plants and microorganisms (Jansson and Persson, 1982; Myrold and Bottomley, 2008). While N uptake from the soil by plants is one of the major mechanisms for long-term N immobilisation (Kuzyakov and Xu, 2013; Orwin et al., 2020), this review focuses on microbial N immobilisation because of its key role in soil N retention (Li et al., 2021).

The availability of N for nitrification is largely determined by the balance between N mineralisation and immobilisation. Generally, this balance is considered to be a function of the metabolic elemental demand of the microbial community and the relative availability of C and N, i.e., C:N ratio (Myrold and Bottomley, 2008; Paterson, 2003). High soil C:N ratios are typically associated with low N mineralisation rates and high N immobilisation rates (Booth et al., 2005). Because decomposition of substrates with a high C:N ratio mineralises less N per unit C, and because the stoichiometric N demand of the heterotrophic microbial community increases in such C-rich conditions, microbial N immobilisation can increase with the soil C:N ratio (Booth et al., 2005; Cleveland and Liptzin, 2007; Fisk et al., 2015; Sterner and Elser, 2002). While an increased C availability should in theory reduce N mineralisation, increase microbial N immobilisation and thus reduce both the availability of N for nitrification and the subsequent risk for N leaching, the evidence from the scientific literature towards this net effect is inconsistent (Fisk et al., 2015; Zhao et al., 2018). For example, past studies have shown that microbial N immobilisation is not always positively associated with the soil C:N ratio (Fisk et al., 2015; Zhao et al., 2018). Therefore, the usefulness of the soil C:N ratio as a predictor for N cycling remains questionable (Bonanomi et al., 2019). This requires further research to investigate the influence of C availability on soil N dynamics (Cao et al., 2021).

2.3 Carbon cycling in grassland ecosystems

The upper 1 m of global grassland soils contain approximately 303 Pg C, which comprises about 20% of the world's total soil C content (Stockmann et al., 2013). Therefore, and because of the spatial extent of grasslands, small changes in grassland soil C stocks can substantially impact atmospheric carbon dioxide (CO_2) concentrations and global C cycling (Conant et al., 2017; Smith, 2008). Grasslands have gained attention increasingly because of their high potential to store C, which could contribute significantly to the United Nations Sustainable Development Goals and other efforts to offset greenhouse gas emissions (Conant et al., 2017; Lal, 2004; Smith, 2008; Soussana et al., 2019; United Nations, 2019).

The potential for grasslands to sequester C is highly dependent on grassland use and management, such as grazing intensity, fertiliser inputs, and grassland plant species used (Conant et al., 2017; McSherry and Ritchie, 2013). The effects of management practices are not always unidirectional (McSherry and Ritchie, 2013; Piñeiro et al., 2010; Trost et al., 2013). For example, grazing can lead to C gains (Reeder and Schuman, 2002; Wang et al., 2016), C losses (Ingram et al., 2008; Klumpp et al., 2009), or to no changes in soil C stocks (Li et al., 2012; Schipper et al., 2014). In addition to the inherent uncertainty in measurements of soil C stock dynamics due to slow changes and high spatial variability at the paddock scale, the effects of management practices on soil C stocks often depend on other factors, such as soil edaphic properties and plant species composition (Baldock et al., 2012; McSherry and Ritchie, 2013; Schipper et al., 2014; Whitehead et al., 2018). However, studying these factors can help to reveal grassland management practices that are more likely to lead to C gains, for example, by introducing certain plant species or adjusting the grazing intensity (Conant et al., 2017; Frasier et al., 2019). Conant et al. (2017) synthesised numerous studies across the globe to compare the effects of improved vs. unimproved grassland management and showed that soil C concentrations were consistently higher under improved management (Figure 2.5). To successfully identify and implement improved grassland management practices that maintain or increase soil C concentrations, research is needed to investigate how management practices affect soil C concentrations and consider spatial and temporal variability (Whitehead et al., 2018).

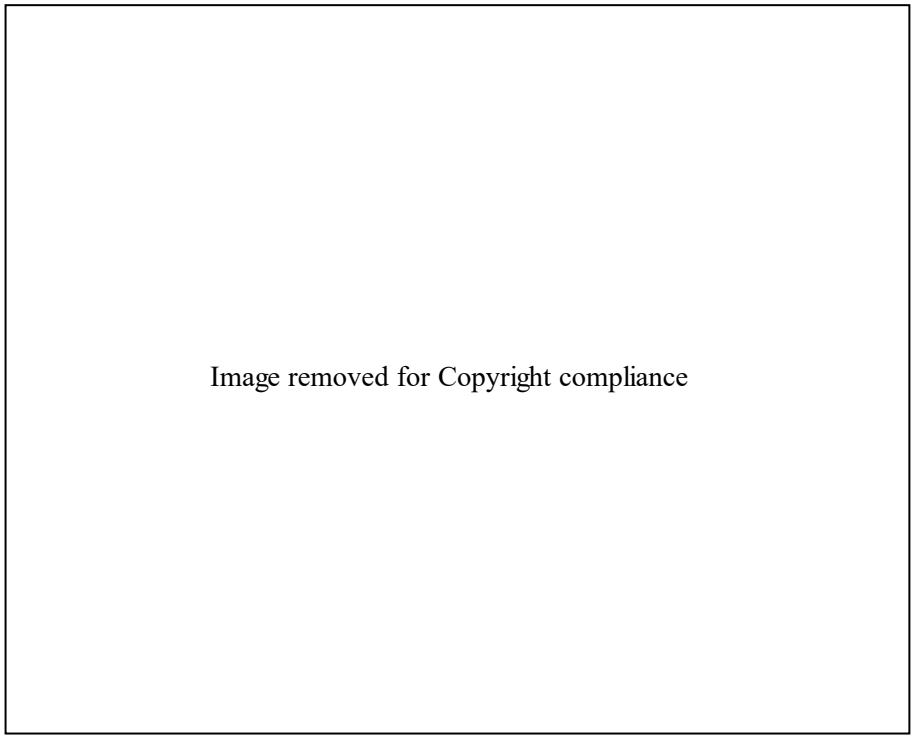

Figure 2.5. Soil C concentrations for grasslands under improved vs. unimproved management practices. Different symbols indicate different types of grassland management change, i.e., conversion of agricultural cultivation to grassland, conversion of native vegetation to grassland, fertiliser addition, fire, improved grazing, and sowing legumes (from Conant et al., 2017).

2.3.1 Carbon inputs to soils through rhizodeposition

Plants link the abiotic with the biotic part of the C cycle by converting CO_2 from the atmosphere to SOC (Pausch and Kuzyakov, 2018). In an extensive review, Pausch and Kuzyakov (2018) showed that on average about 17% of photosynthetically assimilated C by perennial grassland plants was transferred as rhizodeposits to the soil; about 70% of this C was quickly respired, while about 30% of this C remained in the soil after microbial utilisation and decomposition. The vast majority of stabilised SOC is derived from belowground inputs, which can contribute 2 to 13 times more C to SOC formation than aboveground plant litter inputs (Jackson et al., 2017; Sokol et al., 2019). However, the mechanisms regulating the balance between plant C inputs and SOC dynamics are not well understood (Jackson et al., 2017; Jones et al., 2004; Pollierer et al., 2007).

The process where living plant roots release organic C compounds is commonly referred to as rhizodeposition (Kuzyakov and Domanski, 2000), and includes different mechanisms, such as the (1) release of root cap and border cells, (2) release of insoluble mucilage, (3) release of (soluble) root exudates, (4) release of volatile organic C, (5) C flow to symbionts (e.g. arbuscular mycorrhizae), and (6) lysis of senescing root cells (Figure 2.6) (Jones et al., 2009). Rhizodeposits are significant for many soil functions, for example, aggregate formation (Baumert et al., 2018; Six et al., 2004), increasing nutrient availability (Bais et al., 2006; Meier et al., 2017), and regulating soil microbial communities and their activity (Kuzyakov and Blagodatskaya, 2015; Sasse et al., 2018). Since the quantity and quality of rhizodeposits can vary depending on abiotic and biotic conditions, prediction of the effects of rhizodeposition on soil functions is challenging (Figure 2.7) (Badri and Vivanco, 2009; Bais et al., 2006; Hütsch et al., 2002; Jones et al., 2004; Nguyen, 2003).

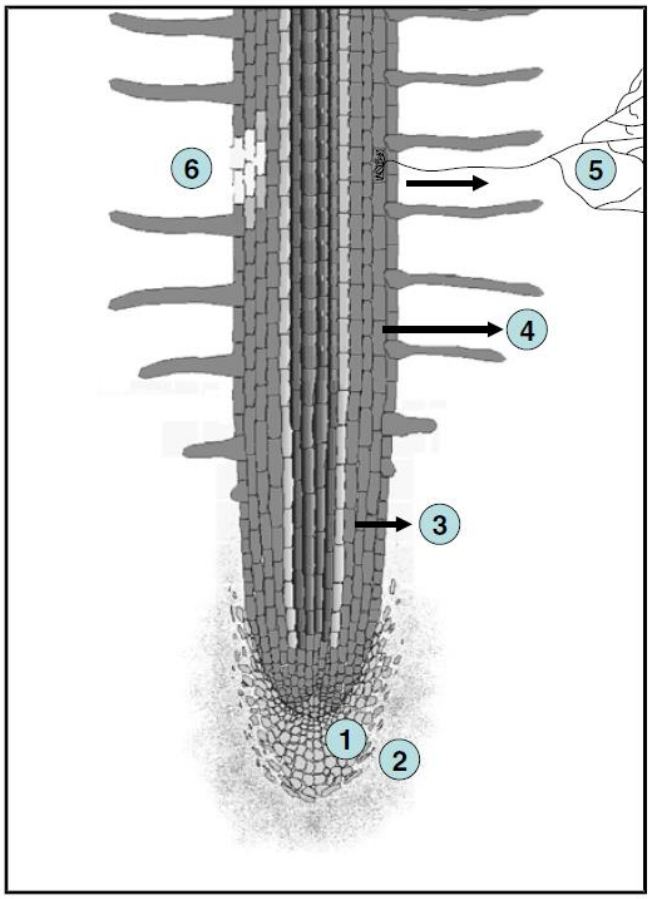

Figure 2.6. Schematic representation of a root with the main mechanisms of rhizodeposition at the respective sites: (1) release of root cap and border cells, (2) release of insoluble mucilage, (3) release of (soluble) root exudates, (4) release of volatile organic C, (5) C flow to symbionts (e.g. arbuscular mycorrhizae), and (6) lysis of senescing root cells (reprinted by permission from Springer Nature, Copyright (2009), Jones et al., 2009).

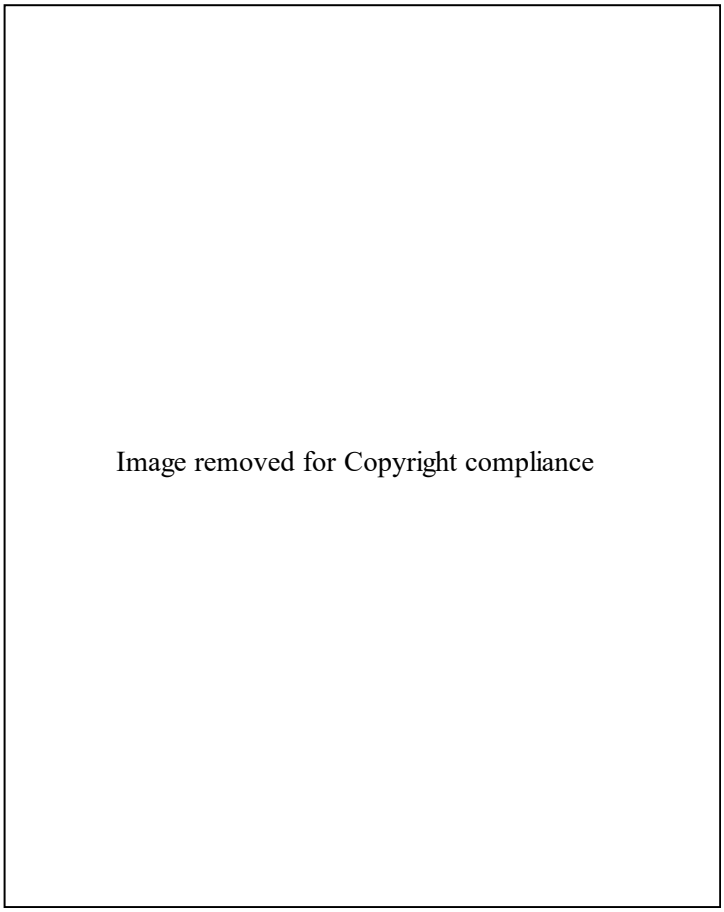

Figure 2.7. Schematic representation of some biotic and abiotic factors of plant and soil that can influence rhizodeposition (from Jones et al., 2004).

One of the factors that influences the amount and composition of rhizodeposits is soil N availability (Bowsher et al., 2018; Nguyen, 2003). Past studies have reported that an insufficient N supply reduces the proportion of amino acids in rhizodeposits, while N addition was associated with an increase in total C rhizodeposition, including the release of carbohydrates and carboxylates (Baptist et al., 2015; Bowsher et al., 2018; Carvalhais et al., 2011; Haase et al., 2007; Henry et al., 2005; Kaštovská et al., 2017). In contrast, there are also reports of decreasing C rhizodeposition with increasing N availability, which are often related to reduced root biomass in N-rich conditions (Chowdhury et al., 2014; Kuzyakov and Domanski, 2000; Nguyen, 2003). These inconclusive results and the limited number of observations emphasise that the effects of N availability on C rhizodeposition are currently not well understood (Liu and Greaver, 2010).

The collection and analysis of rhizodeposits in the soil is difficult, because the root-soil interface is spatially confined and, compared to other soil organic compounds, rhizodeposits are chemically similar, low concentrated, and turned over rapidly by soil microorganisms (Kuzyakov and Domanski, 2000; Pausch and Kuzyakov, 2018). Although there is a broad range of techniques to collect and analyse rhizodeposits, all of them come with limitations that require critical selection of the methods used and

interpretation of results (Oburger and Schmidt, 2016). The use of stable isotopes has been used to trace C through the root-soil-microbe system and to investigate the transformation of root C to SOC (De Deyn et al., 2011; Denef et al., 2009; Pett-Ridge and Firestone, 2017). As an example, the dynamics of C allocation within the plant-soil system can be analysed with $^{13}CO_2$ pulse-labelling, by fumigating plants in an atmosphere enriched with $^{13}CO_2$ for a short period of time (often within hours) (Paterson et al., 2009; Pausch and Kuzyakov, 2018). After photosynthetic uptake, the ^{13}C label is non-uniformly distributed within the plant and accumulates primarily in zones of active growth (Kuzyakov and Domanski, 2000; Paterson et al., 2009). The assumption is that that ^{13}C enriched compounds found in the soil after pulse labelling represent newly assimilated and rhizodeposited C, which can provide information on the fate of recent rhizodeposits in the soil-microbe system (Carbone and Trumbore, 2007; De Deyn et al., 2011; Denef et al., 2009; Olsson and Johnson, 2005; Paterson et al., 2009). Microbial activity and preferential uptake of recent rhizodeposits can be identified directly by analysing the isotopic enrichment of microbial biomarkers, for example phospholipid fatty acids (PLFA) (Haichar et al., 2016; Pett-Ridge and Firestone, 2017; Watzinger, 2015; Yao et al., 2015).

2.3.2 Soil organic matter turnover and stabilisation in soil

Most C entering the soil is relatively rapidly decomposed by soil microorganisms and respired as CO_2, while a fraction can remain in the soil contained as part of SOM for centuries or even millennia (Dungait et al., 2012b; von Lützow et al., 2006). The mechanisms that determine how long C compounds reside in the soil are largely unknown (Kästner and Miltner, 2018; Schmidt et al., 2011), however, recent studies have significantly enhanced our understanding of historic and emerging concepts on SOM formation and turnover (Castellano et al., 2015; Cotrufo et al., 2015, 2013; Dungait et al., 2012b; Lehmann and Kleber, 2015).

Currently, it is understood that relatively stabilised SOM with long residence times consists of low molecular weight organic compounds from plants and microbial cells and forms through mineral associations, including occlusion in aggregates and sorption to minerals, which protect SOM against turnover by reducing microbial accessibility (Angst et al., 2021; Cotrufo et al., 2019, 2013; Kallenbach et al., 2016; Lavallee et al., 2019). The formation of mineral-associated organic matter (MAOM) is regulated in part by the availability of N, because the production of its microbial constituents depends on the quality of the consumed substrate (i.e., substrate or litter C:N ratio) (Castellano et al., 2015; Lavallee et al., 2019). For example, when N availability is high, microbial substrate use and the anabolism:catabolism ratio is high, which leads to greater microbial biomass production and lower respiration losses per unit mass of substrate consumed (Castellano et al., 2015; Poeplau et al., 2019). As a result, large amounts of microbial necromass are produced and can be stabilised. In a meta-analysis, Piñeiro et al. (2010) found that SOM C:N ratios were consistently higher in grazed grasslands than in ungrazed grasslands and hypothesised that SOM formation may be limited by N.

However, while high N availability likely enhances SOM concentrations in many ecosystems (Xu et al., 2021), there is a large variability between sites (Bradford et al., 2008; Khan et al., 2007; Riggs et al., 2015; Ye et al., 2018). For example, SOM concentrations in grassland soils in New Zealand are moderate to high and may be close to C-saturation, so it is unlikely that N addition will lead to further increases (Kirschbaum et al., 2017; Whitehead et al., 2018). This is supported by Chung et al. (2010), who found no significant effect of N inputs on SOM stabilisation for soils rich in SOM, and attributed this finding to potential soil C-saturation. Furthermore, high N availabilities are associated with high N losses, and can thereby offset beneficial effects of C sequestration (Ausseil et al., 2013; Duru et al., 2019; Fornara et al., 2013).

2.4 Coupling of carbon and nitrogen cycles in grassland ecosystems

The C and N cycles in grasslands are tightly coupled through the interaction between the soil and plants (Figure 2.8) (Lemaire et al., 2014; Paterson, 2003; Soussana and Lemaire, 2014). Autotrophic plants couple C and N through biomass growth with photosynthetic CO_2 uptake from the atmosphere and N assimilation via roots (Rumpel and Chabbi, 2019; Soussana and Lemaire, 2014). In the soil, heterotrophic microorganisms require C and N in stoichiometrically constrained proportions to build biomass and SOM, which leads to a close coupling between soil C and N cycles (Cotrufo et al., 2019; Finzi et al., 2011). This strong coupling between C and N in grassland soils can lead to relatively high C and N retention capacities through promotion of soil C storage and reduced accumulation of NO_3^- (Lemaire et al., 2014; Soussana and Lemaire, 2014). However, intensive grassland use can decouple the C and N cycles, thus diminishing C and N retention (Dungait et al., 2012a; Rumpel et al., 2015; Soussana and Lemaire, 2014).

Livestock grazing can lead to a decoupling of C and N cycles by altering stoichiometric interactions from biomass removal and redistribution of elements via urine and dung deposition (Figure 2.8) (Rumpel et al., 2015). About 70% of C consumed by grazing livestock is released as CO_2 and methane (CH_4) to the atmosphere, while 70 to 80% of N taken up is deposited as urine onto the soil surface (Ledgard et al., 2009; Lemaire et al., 2014; Parsons et al., 2013). Hence, only a small proportion of about 20 to 30% of consumed N remains coupled with C and is returned to the soil as dung (Parsons et al., 2013; Soussana and Lemaire, 2014). Furthermore, grazing can decouple C and N cycles within the soil by promoting exploitative root traits that are associated with bacteria-dominated microbial communities, SOM decomposition, and C and N losses (Bardgett, 2017; Bardgett et al., 1998; Klumpp et al., 2009; Recous et al., 2019; Soussana and Lemaire, 2014). These soil C and N losses increase with animal numbers (i.e., stocking density), leading to reduced potentials for C storage and N retention (Ledgard et al., 2009; Soussana et al., 2010; Soussana and Lemaire, 2014). Further understanding of the mechanisms that couple and decouple C and N cycles is crucial to ensure that management practices do not lead to uncontrolled decoupling and C and N losses (Rumpel and Chabbi, 2019).

On the other hand, sustainable grassland management practices can reduce C and N losses by recoupling C and N cycles (Drinkwater and Snapp, 2007; Duru et al., 2019; Soussana and Lemaire, 2014). For example, it has been suggested that the soil C and N cycles can be recoupled through the immobilisation of N by soil microorganisms, stimulated by increased C rhizodeposition (Drinkwater and Snapp, 2007). An increased C rhizodeposition can stimulate growth of the typically C-limited heterotrophic soil microbial community, thereby increasing its stoichiometric N demand and microbial N immobilisation (Abalos et al., 2019; Fisk et al., 2015). However, there is little experimental evidence for this mechanism (Bengtson et al., 2012; Drinkwater and Snapp, 2007) and the consequential effects on, for example, N leaching (Abalos et al., 2019).

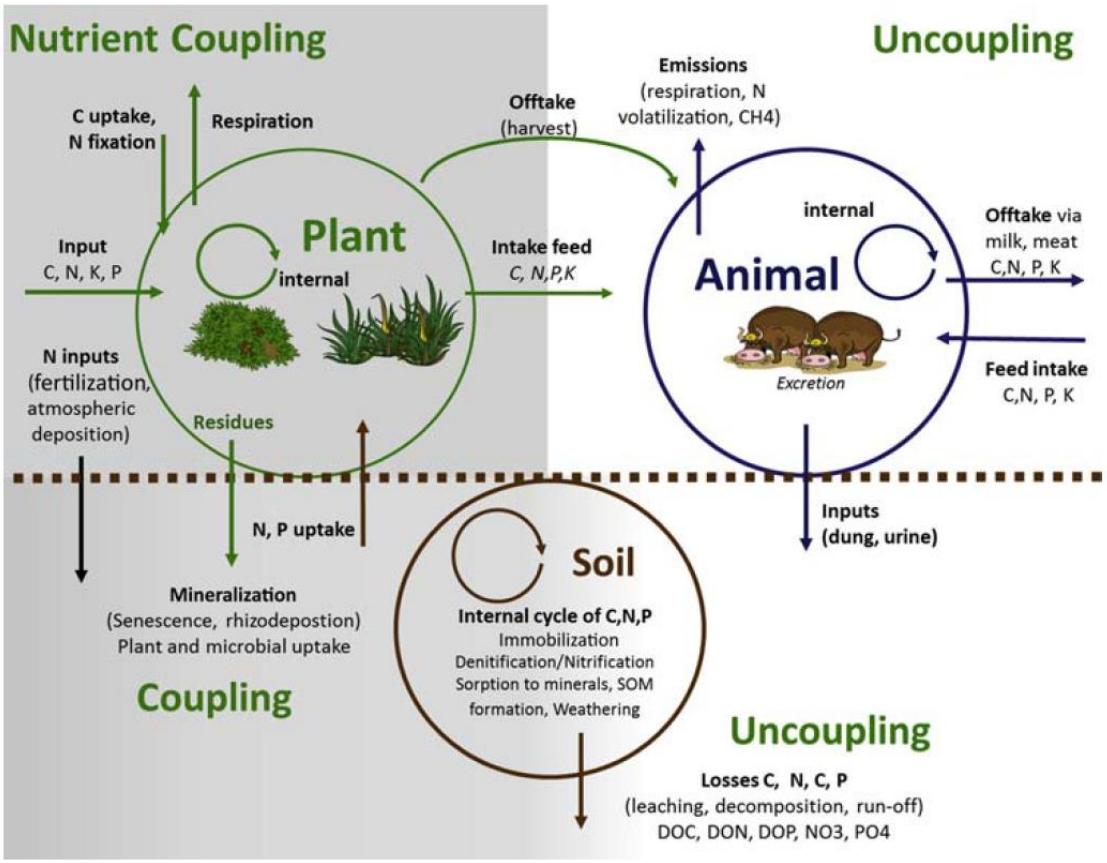

Figure 2.8. Coupling and decoupling of carbon (C), nitrogen (N), and phosphorus (P) cycles in managed grasslands and associated ecosystem processes regulating elemental cycling (reprinted from Vertès et al., 2019, Copyright (2019), with permission from Elsevier).

2.5 Ecological stoichiometry in plant-soil-microbe interactions

Ecological stoichiometry links the metabolic elemental demand of a consumer, for example soil microbial communities, with the elemental composition of substrates, for example SOM (Sterner and Elser, 2002). Stoichiometric elemental limitations can influence microbial growth and elemental cycling in soils substantially, and thus regulate ecosystem functioning (Spohn, 2016; Zechmeister-Boltenstern et al., 2015). However, the link between ecosystem functions and biogeochemical elemental cycling is often missing in conceptual models and this limits understanding of the mechanisms that drive and couple elemental cycles (Buchkowski et al., 2019; Fatichi et al., 2019; Welti et al., 2017). To overcome this barrier, the concept of ecological stoichiometry has been suggested as a framework to link biogeochemistry, trophic interactions, and ecosystem metabolism (Welti et al., 2017).

The soil microbial community uses on average only 30% of C consumed for anabolic processes, while the remaining 70% is respired or invested in extracellular enzymes (Schimel and Weintraub, 2003; Sinsabaugh et al., 2013). In contrast, about 90% of N is on average invested in anabolic processes by the soil microbial community (Mooshammer et al., 2014a). Using the mean soil microbial biomass C:N ratio of approximately 7.6 (Xu et al., 2013), this would suggest that the soil microbial community requires a substrate with a C:N ratio of about 22.8 (Soong et al., 2020). Given that the global mean soil C:N ratio is about 17 (Xu et al., 2013), the soil microbial community is often limited by C (Soong et al., 2020). The inverse is true for litter-decomposing microorganisms, which are typically N-limited as mean global leaf litter C:N ratios are greater than 50 (Yuan and Chen, 2009; Zechmeister-Boltenstern et al., 2015). During litter decomposition, more C is lost than N and P, which results in a decrease of C:N and C:P ratios of decomposing plant litter that approach those for SOM and the soil microbial biomass (Figure 2.9) (Zechmeister-Boltenstern et al., 2015).

Figure 2.9. Changes in global C:N and C:P ratios from live to dead plant materials, and convergence of C:N and C:P ratios from detrital pools towards soil organic matter and soil microbes (from Zechmeister-Boltenstern et al., 2015).

The soil microbial community can adapt to elemental imbalances or stoichiometric elemental limitations, with four strategies representing the main non-exclusive adaptation mechanisms (Mooshammer et al., 2014b):

Firstly, the microbial community exhibits non-homeostatic behaviour and adjusts its biomass stoichiometry to the stoichiometry of its resources (Mooshammer et al., 2014b). Generally, the biomass of the soil microbial community tends towards a characteristic C:N:P stoichiometry of 60:7:1 (Cleveland and Liptzin, 2007), and this tight regulation of the microbial biomass stoichiometry in spite of much more variable substrate stoichiometry is termed 'homeostasis' (Sterner and Elser, 2002). However, individual taxa within the soil microbial community can exhibit a wide range of either homeo- or non-homeostatic behaviours (Mouginot et al., 2014). Hence, non-homeostatic behaviour at the community level is aided by shifts in the soil microbial community composition in response to variable substrate stoichiometries (X. Chen et al., 2014; Cleveland and Liptzin, 2007; Fanin et al., 2013; Heuck et al., 2015; Xu et al., 2013). For example, environments with a high C:N ratio like leaf litter tend to favour fungi (biomass C:N ratio of approximately 5 to 15) over bacteria (biomass C:N ratio of approximately 3 to 6) (Mouginot et al., 2014; Strickland and Rousk, 2010).

Secondly, the microbial community adjusts its production of specific extracellular enzymes to increase the rates of mineralisation and acquisition of the limiting element (Mooshammer et al., 2014b). Thus, the metabolic limitation in the microbial community can become evident through the stoichiometry of enzyme activities, termed 'eco-enzymatic stoichiometry' (Fanin et al., 2016; Sinsabaugh et al., 2009; Sinsabaugh and Follstad Shah, 2012). Globally, the eco-enzymatic stoichiometric C:N:P ratio is well-constrained near a mean of 1:1:1, which is considered to represent the stoichiometric equilibrium between microbial elemental demand and supply (Sinsabaugh et al., 2009).

Thirdly, the microbial community changes its elemental use efficiency so that it excretes elements that are in excess of its requirements and retains those which are limited, independently of the elemental composition of the consumed substrate (Mooshammer et al., 2014b). For example, a substrate rich in N will likely lead to the microbial community becoming C-limited and so C use efficiency is maximal (i.e., high C anabolism:catabolism ratio) (Manzoni et al., 2012). In this situation, the N immobilisation:mineralisation ratio is low and N excess is released. If N becomes limited relative to C, the N use efficiency of the microbial community increases (i.e., high N immobilisation:mineralisation ratio) and excess C is released as overflow respiration, i.e., lower C use efficiency (Manzoni and Porporato, 2009; Schimel and Weintraub, 2003). This shift from net N immobilisation to net N mineralisation is represented by the critical C:nutrient ratio (Manzoni and Porporato, 2009) or threshold elemental ratio (Frost et al., 2006) (Figure 2.10).

Fourthly, the decomposing microbial community obtains N and P from external sources via saprotrophic fungi and N-fixing microorganisms (Mooshammer et al., 2014b). Because N-fixing microorganisms constitute only a small proportion of the decomposing microbial community (Ducey et al., 2013; Jung et al., 2012; Reed et al., 2010), they presumably contribute only marginally to the N supply that is required to alleviate stoichiometric imbalances (Mooshammer et al., 2014b). In contrast, elemental reallocation by saprotrophic fungi can affect the stoichiometric imbalance between decomposers and their substrates significantly (Mooshammer et al., 2014b). The extensive hyphal network of fungi has been shown to efficiently transport C and other elements between sites of different elemental concentrations within the soil or litter as well as between soil and litter (Chigineva et al., 2011; Frey et al., 2003; Osono et al., 2003; Schimel and Hättenschwiler, 2007; Strickland and Rousk, 2010).

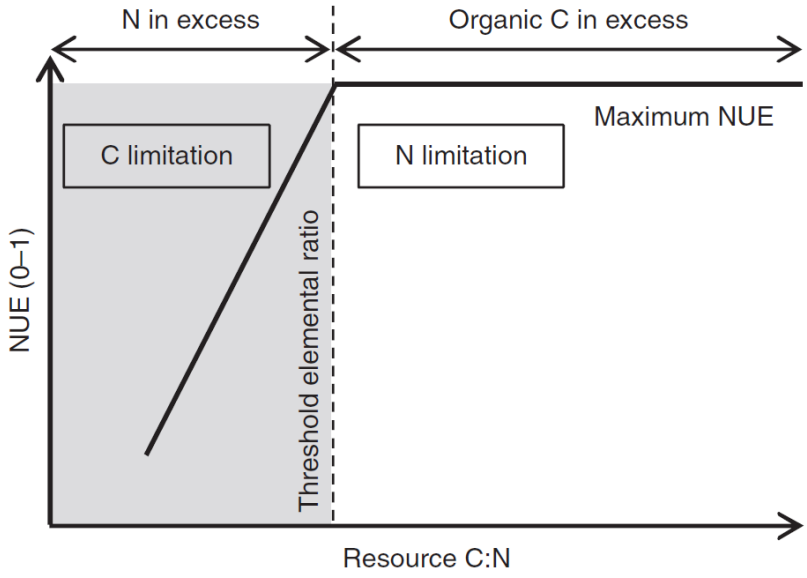

Figure 2.10. Conceptual representation of the regulation of nitrogen use efficiency (NUE) in a homeostatic heterotrophic microbial community. The threshold elemental ratio indicates the critical carbon:nitrogen (C:N) ratio below which N is in excess in relation to microbial N demand (i.e., C limitation). Excess N will be released (net N mineralisation) and microbial NUE decreases. Above the threshold elemental ratio, the microbial community is N-limited and microbial NUE reaches its maximum (net N immobilisation) (from Mooshammer et al., 2014a, reprinted under the Creative Commons CC BY licence).

These different response strategies of the microbial community to stoichiometric imbalances determine microbial activity and community composition, and thereby regulate ecosystem elemental cycling and function (Peñuelas et al., 2012; Sterner and Elser, 2002; Zechmeister-Boltenstern et al., 2015). For example, microbial nutrient limitation has been shown to inhibit soil respiration and reduce SOM accumulation rates (Kirkby et al., 2014, 2013; Spohn and Chodak, 2015). In grassland systems, Schleuss et al. (2021, 2019) demonstrated that adding N induced a stoichiometric imbalance between microbial

biomass and their substrates, and that the homeostatic microbial community responded to this imbalance by adjusting its eco-enzymatic stoichiometry. This impacted functional processes related to C and N cycling, as soil respiration rates decreased while net N mineralisation rates increased (Schleuss et al., 2021, 2019). Understanding these stoichiometrically regulated mechanisms could improve predictions on SOM dynamics and ecosystem functioning in response to management effects and help to identify sustainable management practices (Buchkowski et al., 2019; Fatichi et al., 2019; Soong et al., 2020).

2.6 Summary

There is an urgent need for new grassland management strategies to reduce C and N losses from grazed grasslands in New Zealand (Environment Canterbury, 2018; Jay, 2007; McDowell et al., 2011). This literature review has shown that the identification and development of these strategies is constrained by a limited understanding of the mechanisms that couple and decouple C and N cycles in grassland systems with different management practices (de Vries and Bardgett, 2012; McSherry and Ritchie, 2013; Rumpel and Chabbi, 2019; Soussana and Lemaire, 2014). Although it has been suggested that increased C availability, for example from rhizodeposition, can increase the stoichiometric N demand of the soil microbial community and so increase microbial N immobilisation, there is little experimental evidence to support this (Abalos et al., 2019; Bengtson et al., 2012; Drinkwater and Snapp, 2007; Fisk et al., 2015). Therefore, research is needed to investigate how soil N cycling is impacted by an increased availability of C (Cao et al., 2021).

Rhizodeposits in the soil are difficult to measure and are influenced by various biotic and abiotic factors, such as plant species, N availability for root uptake, and processing by the soil microbial community (Jones et al., 2004; Kuzyakov and Domanski, 2000; Oburger and Schmidt, 2016). Previous findings of the effect of increased N availability in C rhizodeposition for different plant species and of the fate of rhizodeposited C in the soil are often inconsistent (Denef et al., 2009; Liu and Greaver, 2010). To improve predictions of rhizodeposited C on soil functions in grazed grasslands, studies are needed that integrate both the effects of plant species and of increased N availability in investigations of C rhizodeposition and the fate of rhizodeposited C in the soil-microbe system.

Despite increasing recognition that soil microbial elemental limitations can regulate ecosystem functioning significantly by influencing soil elemental cycles (Spohn, 2016; Zechmeister-Boltenstern et al., 2015), the link between biogeochemical elemental cycling and ecosystem functions has often been overlooked in conceptual models (Buchkowski et al., 2019; Fatichi et al., 2019; Welti et al., 2017). The application of ecological stoichiometry to the plant-soil-microbe system could provide a way forward for enhancing knowledge of the mechanisms that regulate and couple elemental cycles and ecosystem processes by linking biogeochemistry, trophic interactions, and ecosystem metabolism (Welti et al., 2017; Zechmeister-Boltenstern et al., 2015).

In summary, this literature review has identified three key knowledge gaps in the scientific literature:

1. How does increased root-derived C supply from different plant species to soil microorganisms affect soil nitrification?

2. How does increased N availability for plant root uptake affect ecosystem C balance, C rhizodeposition, and the fate of rhizodeposited C in the plant-soil-microbe system under different plant species?

3. How does the soil microbial community adapt to stoichiometric elemental limitations under different grassland management practices? What consequences does this have on soil functional processes and soil organic matter concentrations?

The following research chapters will address these knowledge gaps.

Chapter 3

Increased soil nitrogen supply enhances root-derived soil carbon leading to reduced potential nitrification activity

3.1 Abstract

Nitrogen (N) immobilisation by heterotrophic microorganisms is critical for reducing N losses from soils and ensuring a long-term supply of N to plants in grassland ecosystems. The supply of carbon (C) available to soil microbes may stimulate heterotrophic N immobilisation by reducing the availability of ammonium to autotrophic nitrifiers and, hence, for nitrification activity. The main source of available C to soils is rhizodeposition, but its effects on nitrification activity remain unclear as rhizodeposition differs between plant species and varying N availabilities. The aim of this work was to investigate the role of root-derived C on nitrification activity for five different grassland plant species. *Cichorium intybus* (chicory), *Lolium perenne* (perennial ryegrass), *Plantago lanceolata* (ribwort plantain), *Raphanus raphanistrum* and *R. sativus* (wild and cultivated radish), and an unplanted control were grown for nine weeks under controlled environmental conditions and treated either with a low (no urea-N) or a high rate of additional N (550 kg urea-N ha^{-1}). Plant biomass, water-extractable C concentration and ammonia-oxidising bacteria (AOB) abundance increased in the planted high N treatments. The high N addition to planted soils resulted in increased C available for microbial activity and led to decreased potential nitrification activity compared to those for the low N treatments. An increase in water-extractable C concentration was associated with a decrease in potential nitrification activity, suggesting that the increase in available C for microbial activity may have stimulated heterotrophic NH_4^+ uptake and thus N immobilisation.

This study highlights that N addition can be used to manipulate root-derived available C and, with the tight coupling of soil C and N cycling processes, can be used to identify management practices that will promote N retention and reduce losses from grassland soils.

3.2 Introduction

Many grassland ecosystems are limited by nitrogen (N) supply (LeBauer and Treseder, 2008), but management practices are needed to avoid excessive supply from fertiliser additions and livestock urine and dung that can lead to leaching losses and nitrous oxide emissions (Erisman, 2004; Galloway et al., 2008; Schlesinger, 2009). However, identifying effective management practices remains challenging because the mechanisms regulating N cycling and N retention capacities of grassland soils are poorly understood (de Vries and Bardgett, 2016, 2012; Weitzman and Kaye, 2016).

Nitrification of ammonium (NH_4^+) to nitrate (NO_3^-) is a critical step in the regulation of soil NO_3^- concentration, and thus the mobility and leaching susceptibility of N (Canfield et al., 2010). Managing conditions to limit nitrification is likely to result in reduced N leaching losses (Robertson and Vitousek, 2009). Nitrification typically increases when the available NH_4^+ supply exceeds plant N uptake (Robertson and Groffman, 2015), and this often occurs in intensively grazed grasslands with urine deposition, leading to high NO_3^- leaching losses (Crews and Peoples, 2005; Selbie et al., 2015).

A management option to balance N availability and plant demand is to increase immobilisation of excess soil N by soil heterotrophic microorganisms with the subsequent release of mineralised N when plant demand increases (Robertson and Vitousek, 2009). Heterotrophic microorganisms are more competitive for N than autotrophic nitrifying microorganisms (Booth et al., 2005; Dilly, 2005; Sayavedra-Soto and Arp, 2011), and the stoichiometric N demand of heterotrophs can be enhanced by increasing the supply of available carbon (C) substrates (Booth et al., 2005; Cleveland and Liptzin, 2007; Hart et al., 1994a; Sterner and Elser, 2002). Available C compounds that are microbially accessible can be derived readily from decomposed organic matter by microbial and enzymatic processes (R. Chen et al., 2014; Dungait et al., 2012b; Marschner and Kalbitz, 2003). Increasing the available C supply (i.e., increasing the soil C:N ratio) has been shown to decrease autotrophic nitrification activity and reduce the risk of N leaching (Fisk et al., 2015).

Plantago lanceolata (ribwort plantain) and *Raphanus raphanistrum* (wild radish) have been identified for their potential to reduce soil nitrification activities (Massaccesi et al., 2015; O'Sullivan et al., 2017). However, it is unclear if this effect is attributable to the root release of allelochemicals, including biological nitrification inhibitors (BNI), that suppress nitrification activities by affecting nitrifying microbial or enzymatic processes, or to an increase in microbial N immobilisation resulting from increased C rhizodeposition (Carlton et al., 2019; Cong and Eriksen, 2018; Coskun et al., 2017b, 2017a; Subbarao et al., 2015). Rhizodeposits can amount to 20 to 60% of net C assimilated by plants, equivalent to about 800 to 4 500 kg C ha^{-1} annually (Neumann and Römheld, 2012), and the rate and composition of rhizodeposition varies between plant species and under different conditions, such as soil fertility (Badri and Vivanco, 2009; Bais et al., 2006; Jones et al., 2004; Nguyen, 2003). Previous studies have shown that plant N deficiency can reduce the exudation of amino acids, while N addition leads to an

increase in rhizodeposition, including exudation of highly available C compounds, such as carboxylates and sugars (Bowsher et al., 2018; Carvalhais et al., 2011; Haase et al., 2007). Although it has been shown that soil heterotrophic microorganisms can rapidly assimilate rhizodeposited C (Bahn et al., 2013), the effects of C rhizodeposition on soil nitrification remain unknown, due to the variability in rhizodeposition between species, priming effects, and limited observations of the effects of N availability on rhizodeposits (Bowsher et al., 2018; Gärdenäs et al., 2011).

The aim of this study was to investigate the effects of increased available C supply from plant roots on the regulation of soil nitrification activities for different plant species. The first hypothesis was that soil C availability would differ between plant species, and this would affect the abundance of ammonia-oxidising microorganisms and nitrification activities. The second hypothesis was that increased soil N supply would increase C availability, with subsequent decreases in nitrification activity. To test these hypotheses, five grassland species with different root characteristics were grown in microcosms with no and high addition of urea-N. Changes in shoot and root biomass, shoot N concentration and content, soil pH, concentrations of total soil organic C and N, available C and mineral N, abundance of ammonia-oxidising microorganisms, and net nitrification activities associated with plant species, C availability and N addition were investigated.

3.3 Materials and Methods

3.3.1 Site description, soil sampling and experimental design

Topsoil was collected to a depth of 150 mm from an irrigated ryegrass (*Lolium perenne* L.)-white clover (*Trifolium repens* L.) grassland at the Lincoln University Research Dairy Farm (LURDF), Lincoln, New Zealand (latitude 43.640° S, longitude 172.463° E; 14 m above sea level). The site was not grazed and no fertilizer was applied for three years prior to this study. The soil was a Templeton silt loam (Typic Immature Pallic soil (New Zealand Soil Classification) (Hewitt, 2010); Udic Haplustept (USDA) (Soil Survey Staff, 2014)) with a pH ($CaCl_2$) of 4.90 and an organic matter concentration of 44 g kg^{-1}. Soil mineral N concentrations comprised 13 mg NH_4^+-N kg^{-1} and 9 mg NO_3^--N kg^{-1}.

After collection, the soil was sieved (≤ 4 mm), homogenised and 980 g ± 1 g was packed into PVC microcosms (65 mm diameter, 240 mm depth) at a bulk density of 1.0 Mg m^{-3}. In the centre of each microcosm, one seed of one of the following species was planted: *Cichorium intybus* L. cv. 'Choice' (chicory), *Lolium perenne* L. cv. 'Prospect' (perennial ryegrass), *Plantago lanceolata* L. cv. 'Tonic' (ribwort plantain), *Raphanus raphanistrum* L. (wild radish), or *Raphanus sativus* L. cv. 'Saxa 2' (cultivated radish). An unplanted soil treatment was included as a control. These plant species were selected because of their contrasting root structures and distinct effects on soil N dynamics (Massaccesi et al., 2015; O'Sullivan et al., 2017).

The microcosms were arranged in a completely randomised design in a plant growth chamber (Fitotron HGC 1514, Weiss Gallenkamp, UK). The plants were grown under controlled conditions: 16 h photoperiod, air temperature 22 °C, photosynthetically active irradiance of 500 µmol m^{-2} s^{-1} at canopy level and 70% relative humidity. Daily watering, supplemented with a weekly adjustment of gravimetric soil moisture content to achieve 60 to 80 % water holding capacity (WHC), ensured sufficient water supply for plant growth and avoided drainage. Three weeks after the seeds were sown, a top dressing of superphosphate at a rate of 30 kg P ha^{-1} and ammonium sulphate ($(NH_4)_2SO_4$) at a rate of 50 kg N ha^{-1} was applied to each microcosm.

Plants were grown initially for five weeks to allow enough time for the root system to develop. Then, two N treatments were applied to the microcosms. Half of the microcosms received urea ($CO(NH_2)_2$) at a rate of 229 mg N kg $soil^{-1}$, applied in solution to simulate a N loading rate similar to that of a urine patch from dairy cattle of 550 kg N ha^{-1} (Selbie et al., 2015), hereafter referred to as the 'high N treatment'. The other half of the microcosms received the same volume of water but without urea. This treatment is hereafter referred to as the 'low N treatment', as low amounts of N from the initial soil N content and the early $(NH_4)_2SO_4$ fertiliser application were expected to have remained in the soil. Mineralisation of urea to NH_4^+, which could have limited NH_4^+ availability for nitrification, was considered negligible because urea in soils is typically hydrolysed within a few days after surface application (Burton and Prosser, 2001; Cabrera et al., 1991; Sigurdarson et al., 2018). In total, there were 48 microcosms, comprised of four replicate microcosms for each plant species and N treatment.

After applying the urea-N treatment, the plants were grown for another four weeks to ensure that the ammonia-oxidising microbial community had enough time to establish (Di et al., 2009). Following a total incubation time of approximately nine weeks after the seeds were sown, the microcosms were sampled destructively. After removing the soil and plants, soil samples were collected from the rhizosphere (soil adjacent to the roots) using sterilised spatulas and stored at -80 °C for subsequent DNA extraction. The shoots, roots, and bulk soil were separated carefully, the soil was sieved (≤ 4 mm), homogenised and stored at 4 °C in dark conditions until further processing.

3.3.2 Plant analyses and measurements of soil chemical properties

Plant shoots and carefully washed roots were dried at 60 °C for 72 h and then weighed. After grinding, the dried shoots were analysed to determine the C and N concentrations by dry combustion on an elemental analyser (Elementar Vario-Max CN Elemental Analyser, Elementar GmbH, Hanau, Germany). Shoot N contents were calculated by multiplying shoot N concentration with shoot biomass. Root C and N concentrations were not measured.

Gravimetric soil water content was determined from the decrease in mass from fresh soil after drying at 105 °C for 24 h. Soil pH was measured in 0.01 M $CaCl_2$ (1:2.5 w:v). For soil mineral N concentration, ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 2 M KCl (1:10 w:v) from a fresh subsample (Rayment and Lyons, 2011), and concentrations measured using flow injection analysis (FOSS FIAstar 5000, Foss Tecator AB, Hoganas, Sweden). Total organic C (C_t) and total N (N_t) were analysed by dry combustion on an elemental analyser (Elementar Vario-Max CN Elemental Analyser, Elementar GmbH, Hanau, Germany). For determining water-extractable C concentrations (C_{we}), 3.0 ± 0.05 g dry soil equivalent was eluted with 30 mL deionised water (1:10 w:v), centrifuged (3000 × *g* for 20 min), filtered (0.45 µm) (Ghani et al., 2003) and analysed for dissolved organic C (Shimadzu TOC Analyser model 5000A with ASI-5000A, Shimadzu Oceania Pty Ltd., Sydney, Australia).

3.3.3 Carbon availability index

Calculations of the carbon availability index (I_C), which can be used to indicate the proportion of available organic C for microbial use (Parkinson and Coleman, 1991), were determined from the ratio of measurements of soil basal respiration rate (R_{basal}) and substrate induced respiration rate (R_{SI}) (Cheng et al., 1996; Gershenson et al., 2009; Gutiérrez-Girón et al., 2015), using a modified method from Anderson and Domsch (1978). Briefly, 4.0 ± 0.05 g fresh soil was incubated with 0.3 mL deionised water in sealed glass vials in dark conditions at 25 °C for 1 h. The increase in CO_2 concentrations in the headspace between the start and end of the incubation period were measured and used to calculate R_{basal}. This was then repeated after adding glucose at 10 mg g^{-1} (oven-dry soil basis) to a replicate soil sample for determination of R_{SI}. Gas samples from the headspace were taken with a syringe and injected through a CO_2-free air stream into a calibrated infra-red gas analyser (Model LI-7000, LI-COR Inc., Lincoln, NE, USA).

3.3.4 Potential nitrification activity

Potential nitrification activity (N_p), which represents the potential enzyme activity for ammonium oxidation (Kandeler et al., 2011), was estimated after destructive sampling by chlorate inhibition (Belser and Mays, 1980) following a modified procedure of Hart et al. (1994b). In brief, 15 ± 0.5 g fresh soil was placed into an Erlenmeyer flask containing a 100 mL mixture of 1.5 mM NH_4^+ and 1 mM phosphate buffer with a pH adjusted to 7.2 ± 0.1 and added 1.1 g L^{-1} sodium chlorate ($NaClO_3$) as a selective inhibitor of nitrite (NO_2^-) oxidation to nitrate (NO_3^-) (Belser and Mays, 1980). The soil slurry was incubated on a horizontal shaker (115 rpm) at 20 °C for 24 h. At 2, 6, 20, and 24 h after the start of the incubation, 10 mL aliquots with a consistent soil:solution ratio were removed from the flask and centrifuged for 10 min. The supernatant was mixed with sulphanilamide and

N-1(1-naphtyl)ethylenediamine dihydrochloride (NED) for colour development (Griess reaction) and the NO_2^- concentration analysed colorimetrically at 540 nm. The slope of the linear change in NO_2^--N concentration during the incubation time was used to calculate N_p.

3.3.5 DNA extraction and real-time qPCR

Total genomic DNA was extracted in triplicate from 0.25 g ± 0.001 g rhizosphere soil using a NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany). After homogenising the sample mechanically and lysing the cells with a buffer solution, the membrane-bound DNA was washed and extracted, eluted in 100 µL of 5 mM Tris/HCl (pH 8.5) and diluted with filtered and UV-sterilised ultra-pure water (1:10 v/v) to minimise potential inhibition of polymerase chain reaction (PCR). All samples were stored at -20 °C prior to real-time quantitative polymerase chain reaction (qPCR) analysis.

The abundance of the *amoA* genes for ammonia oxidisers were estimated from the qPCR analysis following procedures described by Di et al. (2009) using the primer pairs amoA-1F (5'-GGGGHTTYTACTGGTGGT-3') (Stephen et al., 1999) and amoA R-i (5'-CCCCTCNGNAAANCCTTCTTC-3') (Hornek et al., 2006) for bacterial *amoA* genes and Arch-amoAF (5'-STAATGGTCTGGCTTAGACG-3') and Arch-amoAR (5'-GCGGCCATCCATCTGTATGT-3') (Francis et al., 2005) for archaeal *amoA* genes. A robotic liquid handling system (CAS-1200, Corbett Life Science, Mortlake, Australia) was used to prepare the qPCR setup automatically and all reactions were performed on a Rotor-Gene™ 6000 real-time rotary analyser (Corbett Life Science, Mortlake, Australia). The thermocycling conditions of the qPCR and the specific primer combinations are shown in Table A.1.1 (Appendix A.1). The total volume of 16 µL qPCR reaction mixture comprised a dilution of 8 µL 2× SYBR® Premix Ex Taq™ (TaKaRa, Japan), 0.4 µL of each primer, and 1.5 µL of diluted DNA extract. After each assay, a melting curve analysis ranging from 72 to 99 °C ensured that the melting temperature cycles only caused amplification of the targeted genes.

For standard curve development, the PCR amplicons from extracted DNA samples were purified with a PCR clean-up kit (Axygen Scientific, USA) and the PCR product concentration determined with a Qubit™ fluorometer (Invitrogen, USA). Standard curves were generated based on quantified PCR product in a series of 1:10 dilutions after real-time qPCR with the same thermocycling conditions (Table A.1.1, Appendix A). Amplification efficiencies of 100% were obtained for both AOA and AOB *amoA* ($R^2 \geq 0.995$). Copy numbers for the *amoA* genes per unit mass of dry soil were calculated to estimate the abundances of AOA and AOB.

3.3.6 Statistical analyses

Plant and soil properties (root biomass, shoot biomass, pH, N_p, I_C, AOA and AOB abundance, shoot N concentration, C_t, C_{we}, N_t, NH_4^+-N, and NO_3^--N) were analysed using two-way factorial analysis of variance (ANOVA) with N application rate (2 levels) and plant species (5 levels) as factors, and included possible interactions. Where there were significant differences ($P < 0.05$) between group means, Tukey HSD post-hoc tests were conducted for multiple comparisons of all plant species and N application effects. Multiple comparisons were carried out with the '*multcomp*' package in R to correct for multiplicity (i.e., control type I error) while making many simultaneous inferences (Hothorn et al., 2008). These differences were reported using confidence intervals (95% CI). Results from the unplanted control soil were excluded from statistical analyses that compared plant species effects but included for comparisons between planted and unplanted soils.

Prior to using the ANOVA, assumptions of normality of the residuals and homoskedasticity were assessed by visual inspection of residual plots and plots of predicted vs. observed values. If the variance violated the assumption of homoskedasticity, linear models were refitted using the sandwich estimator of the covariance matrix (Zeileis, 2004), which is consistent for cases of heteroskedasticity. Since I_C is derived from a ratio, I_C was log-transformed to correct for skewness (Koricheva et al., 2013). Spearman's rank correlations were used to investigate associations between two variables.

Potential relationships between N_p and soil variables were investigated using linear regression, initially selecting between competing and sometimes co-linear predictor variables and then comparing models with likelihood ratio tests (if nested) and Akaike Information Criterion (AIC). Residual checks were used throughout. Values of the parameters from these models were reported with 95% confidence intervals (95% CI). All statistical analyses were undertaken using R version 3.6.0 (R Core Team, 2019).

3.4 Results

3.4.1 Plant properties

Shoot biomass for *L. perenne, P. lanceolata*, and *R. raphanistrum* increased in the high N treatment by 440, 217, and 232% compared to the low N treatment, respectively, while there were no significant differences in shoot biomass between N treatments for *C. intybus* and *R. sativus* (Table 3.1). Root biomass was similar between N treatments for all species except for *L. perenne*, where the increase in root biomass was 283% in the high N treatment. Although *L. perenne* responded to the high N treatment with the largest increase in biomass compared to that for the other species, the concentration of N in its shoot overall remained unchanged. In contrast, shoot N concentrations for *C. intybus*, *P. lanceolata*, and *R. sativus* in the high N treatment increased by 251, 205, and 186% relative to those for the low N treatment, respectively.

The shoot N content, an estimate of N uptake for shoot biomass, increased significantly for all species in the high N treatment relative to the low N treatment. *Lolium perenne* showed the highest N uptake, with 47.5% of added N in the high N treatment measured in the shoot. In contrast, *R. sativus* showed the lowest N uptake, with 19.4% of the N added in the high N treatment measured in shoot biomass, while plant uptake for *C. intybus, P. lanceolata,* and *R. raphanistrum* was 34.1, 40.3, and 30.7% of added N in the high N treatment.

Table 3.1. Shoot and root biomass, shoot N concentration and content of plant species under low and high N treatments. Data are mean values ± standard error, n = 4. Different letters indicate significant differences among species and N treatments ($P < 0.05$). Values for shoot N content are expressed as mg N per total mass of shoot dry matter.

Species	N treatment	Shoot biomass	Root biomass	Shoot N concentration	Shoot N content
		g dry matter	g dry matter	mg N g dry matter^{-1}	mg N shoot^{-1}
C. intybus	low	3.92 ± 0.81^{bd}	7.50 ± 1.31^{bc}	5.73 ± 0.61^{e}	21.8 ± 3.25^{d}
	high	5.37 ± 0.64^{bc}	11.90 ± 1.21^{b}	14.40 ± 0.79^{b}	76.5 ± 6.81^{a}
L. perenne	low	2.00 ± 0.47^{de}	8.30 ± 3.49^{bc}	9.95 ± 2.01^{be}	17.0 ± 1.68^{d}
	high	8.80 ± 0.83^{a}	23.50 ± 4.46^{a}	12.30 ± 1.07^{bc}	106.5 ± 7.66^{b}
P. lanceolata	low	2.94 ± 0.21^{cde}	9.21 ± 2.88^{bc}	6.95 ± 0.83^{de}	20.5 ± 2.72^{d}
	high	6.37 ± 0.34^{ab}	9.96 ± 1.22^{bc}	14.28 ± 0.57^{b}	90.5 ± 4.25^{ab}
R. raphanistrum	low	3.99 ± 0.93^{bd}	1.09 ± 0.27^{c}	6.13 ± 0.49^{de}	23.3 ± 4.03^{d}
	high	9.24 ± 0.92^{a}	4.47 ± 0.64^{bc}	7.28 ± 0.78^{cde}	69.0 ± 13.39^{abc}
R. sativus	low	0.45 ± 0.06^{e}	5.43 ± 0.37^{bc}	10.95 ± 1.03^{bd}	4.8 ± 0.25^{e}
	high	2.22 ± 0.38^{de}	8.60 ± 0.37^{bc}	20.40 ± 1.56^{a}	44.0 ± 5.29^{c}

3.4.2 Soil chemical properties

Soil NH_4^+-N and NO_3^--N concentrations in planted soils were higher in the high N treatment compared with those in the low N treatment by 0.705 mg kg^{-1} (95% CI, 0.430 to 0.981; $P < 0.001$) and 2.10 mg kg^{-1} (95% CI, 0.41 to 3.78; $P = 0.010$), respectively (Table 3.2). Mean NO_3^--N concentrations in the planted soils were significantly lower than those in the unplanted control soils. The NO_3^--N concentrations in planted soils were higher in the high N treatment under *C. intybus, L. perenne,* and *P. lanceolata* but slightly lower under *R. raphanistrum* and *R. sativus,* when compared with the low N treatment. These differences were only significant for *L. perenne*. The NH_4^+-N concentrations were higher in the high N treatment for all species, but only significantly higher for *L. perenne*.

Overall, C_t and N_t in planted soils were lower in the high N treatment by 1.93 g kg^{-1} (95% CI, -2.57 to -1.29; $P < 0.001$) and 0.161 g kg^{-1} (95% CI, -0.222 to -0.101; $P < 0.001$) compared to values in the low N treatment, respectively (Table 3.2), equivalent to a decrease of 3.17–11.7% for C_t and 1.45–11.9% for N_t.

Soil pH in the planted soils was 0.262 units (95% CI, -0.305 to -0.219; $P < 0.001$) lower in the high N treatments than that in the low N treatments (Table 3.2). Further, soil pH under *L. perenne* was 0.157 units higher (95% CI, 0.089 to 0.225; $P < 0.001$) than the soil pH for the other species.

Table 3.2. Soil pH, concentrations of total organic carbon (C_t), total nitrogen (N_t), ammonium-nitrogen (NH_4^+-N), and nitrate-nitrogen (NO_3^--N) in the soils under the different plant species and N treatments. Data are mean values ± standard error, n = 4. Different letters indicate significant differences among species and N treatments ($P < 0.05$). Values for C_t, N_t, NH_4^+-N and NO_3^--N concentrations are expressed as per unit dry mass of soil.

Species	N treatment	NH_4^+-N	NO_3^--N	C_t	N_t	pH
		mg kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	
C. intybus	low	0.56 ± 0.07^{de}	0.85 ± 0.56^{cde}	23.3 ± 0.3^{ac}	2.12 ± 0.03^{ac}	4.57 ± 0.03^{bc}
	high	1.57 ± 0.49^{ace}	4.10 ± 3.04^{bcde}	22.6 ± 0.1^{bc}	2.09 ± 0.03^{ac}	4.37 ± 0.04^{de}
L. perenne	low	0.71 ± 0.02^{cd}	0.40 ± 0.03^{d}	24.0 ± 1.0^{ac}	2.18 ± 0.05^{ac}	4.74 ± 0.05^{a}
	high	1.65 ± 0.17^{a}	5.20 ± 1.65^{c}	22.3 ± 0.1^{bc}	$2.04 \pm 0.05b^{c}$	4.51 ± 0.03^{cd}
P. lanceolata	low	0.55 ± 0.02^{e}	0.26 ± 0.02^{e}	25.1 ± 0.5^{a}	2.25 ± 0.05^{ab}	4.56 ± 0.02^{bc}
	high	1.05 ± 0.22^{ace}	2.86 ± 2.00^{cde}	22.3 ± 0.2^{bc}	2.12 ± 0.7^{ac}	4.34 ± 0.02^{e}
R. raphanistrum	low	0.94 ± 0.11^{bc}	0.79 ± 0.22^{cde}	24.0 ± 0.6^{ac}	2.30 ± 0.03^{a}	4.60 ± 0.02^{ac}
	high	1.53 ± 0.28^{ab}	0.73 ± 0.23^{cde}	22.4 ± 0.6^{bc}	2.07 ± 0.05^{bc}	4.30 ± 0.03^{e}
R. sativus	low	0.75 ± 0.15^{bce}	0.58 ± 0.09^{cd}	24.4 ± 0.4^{ab}	2.26 ± 0.03^{ab}	4.61 ± 0.03^{ac}
	high	1.24 ± 0.22^{ac}	0.48 ± 0.09^{de}	21.5 ± 0.2^{c}	1.98 ± 0.04^{c}	4.25 ± 0.04^{e}
Unplanted	low	0.73 ± 0.07^{bce}	12.43 ± 1.45^{b}	24.5 ± 0.8^{ab}	2.22 ± 0.06^{ab}	4.66 ± 0.04^{ab}
control	high	0.96 ± 0.10^{bc}	69.21 ± 8.84^{a}	22.6 ± 0.6^{ac}	2.23 ± 0.05^{ab}	4.26 ± 0.02^{e}

3.4.3 Available soil carbon concentrations

The mean values for C_{we} in the planted soils was 23.3 µg g^{-1} (95% CI, 17.2 to 29.4; $P < 0.001$) higher in the high N treatment than that in the low N treatment (Figure 3.1). There were no significant differences in C_{we} between plant species, whereas C_{we} in the unplanted control was significantly lower than the value in the planted soils for the high N treatment.

Measurements of I_C were 0.703 units (95% CI, 0.477 to 0.929; $P = 0.0109$) higher in the high N treatment than those in the low N treatment (Figure A.1.1, Appendix A.1), and I_C was correlated with C_{we} ($\rho = 0.434$, $P = 0.0055$). There were significant differences in I_C between plant species, but measurements of I_C were compromised by varying soil water contents of the samples ($\rho = 0.480$, $P < 0.001$).

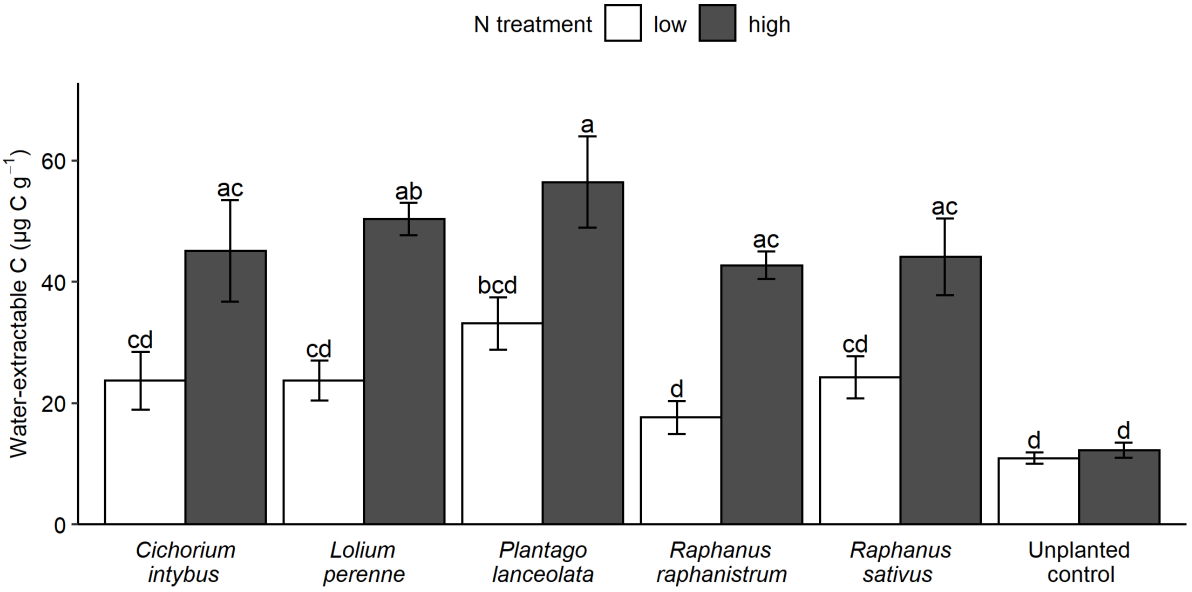

Figure 3.1. Mean water-extractable C concentrations (C_{we}) in the soils for all plant species and controls with high and low N treatments. Error bars represent standard errors, n = 4. Different letters indicate significant differences among plant species or controls and N treatments ($P < 0.05$). Values are expressed as per unit dry mass of soil.

3.4.4 Ammonia-oxidising archaea and bacteria abundances

The abundance of AOA *amoA* gene copies exceeded those of AOB in all treatments. The AOA abundance ranged from 5.7 × 10^6 to 13.6 × 10^6 *amoA* gene copies g^{-1} (Figure 3.2A), while the AOB abundance was between 1.6 × 10^6 and 9.1 × 10^6 *amoA* gene copies g^{-1} (Figure 3.2B). There were no significant differences in AOA abundance related to plant species or N treatment. In contrast, the high N treatment was associated with an increase in AOB abundance by 4.8 × 10^6 *amoA* gene copies g^{-1} (95% CI, 3.5 × 10^6 to 6.1 × 10^6; $P < 0.001$) compared to the low N treatment. There was no significant plant species effect.

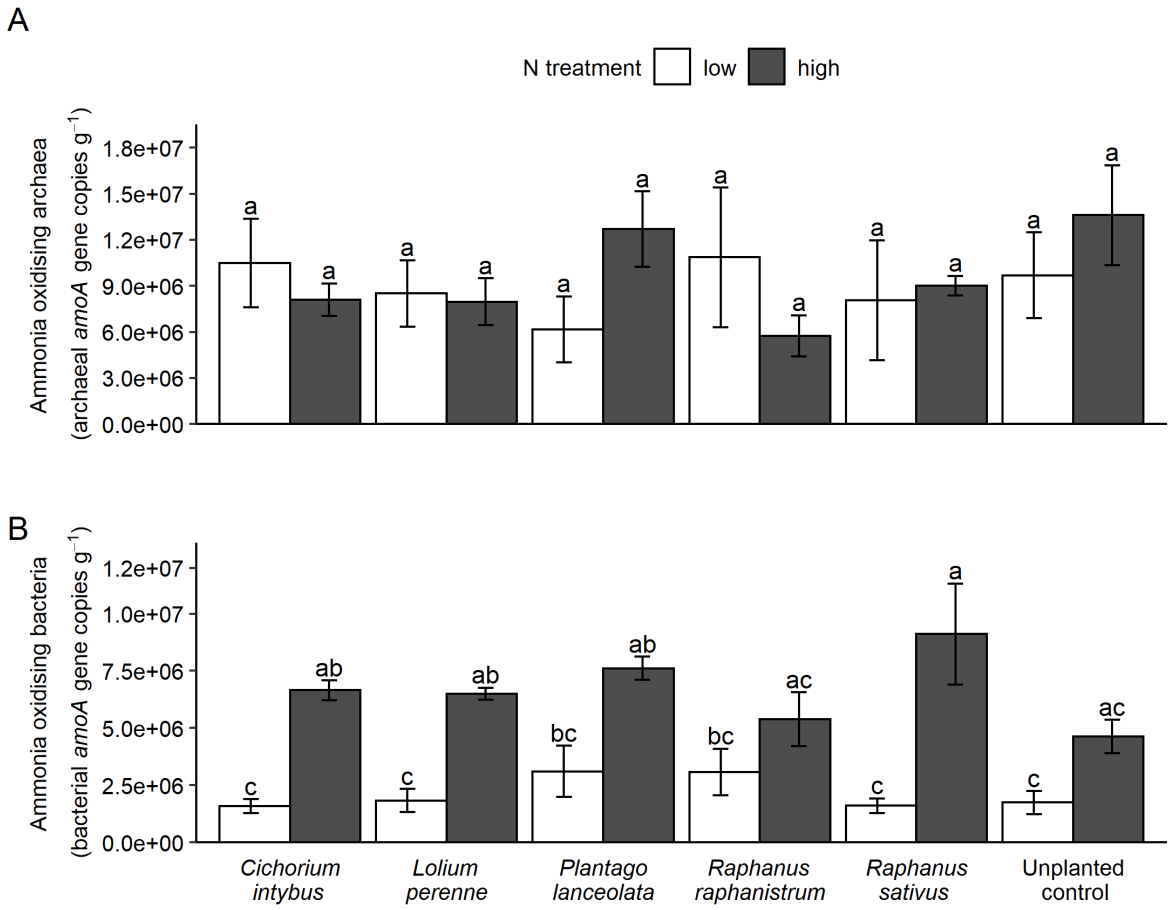

Figure 3.2. Mean *amoA* gene copy abundances of ammonia oxidising archaea (AOA) (A) and bacteria (AOB) (B) in the soils under different plant species and N treatments. Error bars represent standard errors, n = 4. Different letters indicate significant differences among plant species and N treatments ($P < 0.05$). Values are expressed as per unit dry mass of soil.

3.4.5 Potential nitrification activity

Potential nitrification activity (N_p) decreased by 1.36 ± 0.14 µg NO_2^--N g^{-1} day^{-1} (95% CI, -1.64 to -1.07; $P < 0.001$) in the high N treatment relative to that for the low N treatment. This decrease was greatest for *P. lanceolata*, amounting to almost half the N_p (45.8%) in the high N treatment compared to that for the low N treatment (Figure 3.3). For the other species, the decrease in N_p ranged from 26.8 to 33.0% in the high N treatments compared to that for the low N treatments.

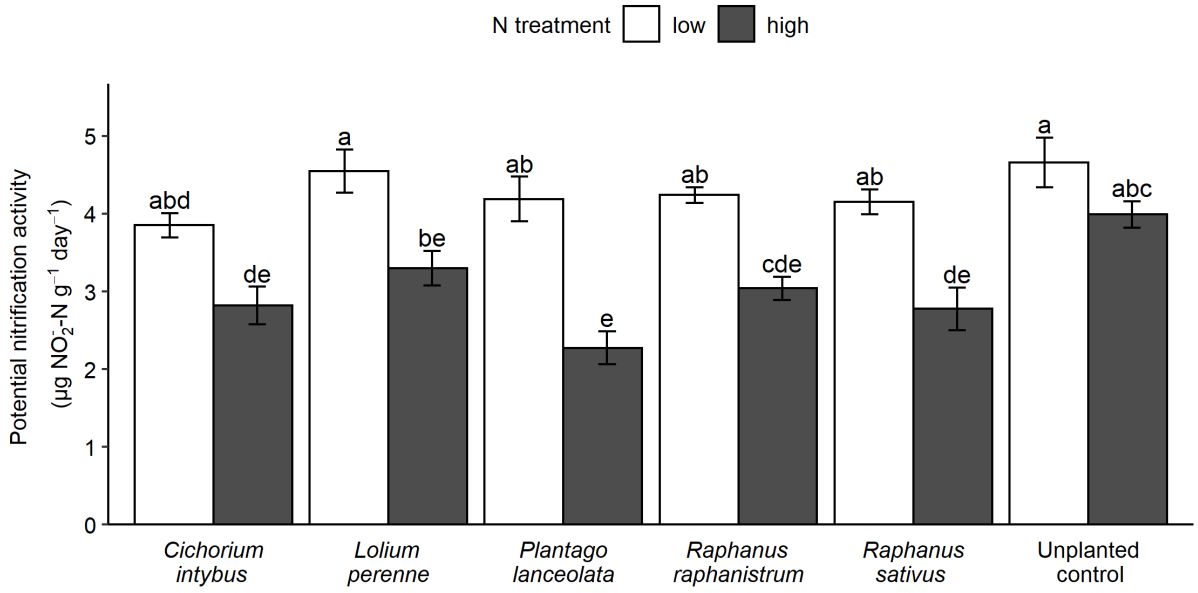

Figure 3.3. Mean potential nitrification activity (N_p) in the soils under different plant species and N treatments. Error bars represent standard errors, n = 4. Different letters indicate significant differences among plant species and N treatments ($P < 0.05$). Values are expressed as per unit dry mass of soil.

There was a negative linear relationship between C_{we} and N_p (RMSE = 0.637 µg NO_2-N g^{-1} day^{-1}, $R^2 = 0.406$, $P < 0.001$), with a slope of -0.0343 µg NO_2^--N g^{-1} day^{-1} (95% CI: -0.048 to -0.021; $P < 0.001$) (Figure 3.4). This negative relationship becomes evident when comparing the N_p:C_{we} ratio between the two N treatments, as C_{we} increased in the high N treatment (Figure 3.5A) while N_p decreased (Figure 3.5B) compared to values for the low N treatment. The N_p:C_{we} ratio was 66.7% lower in the high N treatment (0.064 µg NO_2^--N $µg^{-1}$ C day^{-1}) than that in the low N treatment (0.19 µg NO_2^--N $µg^{-1}$ C day^{-1}) (Figure 3.5C).

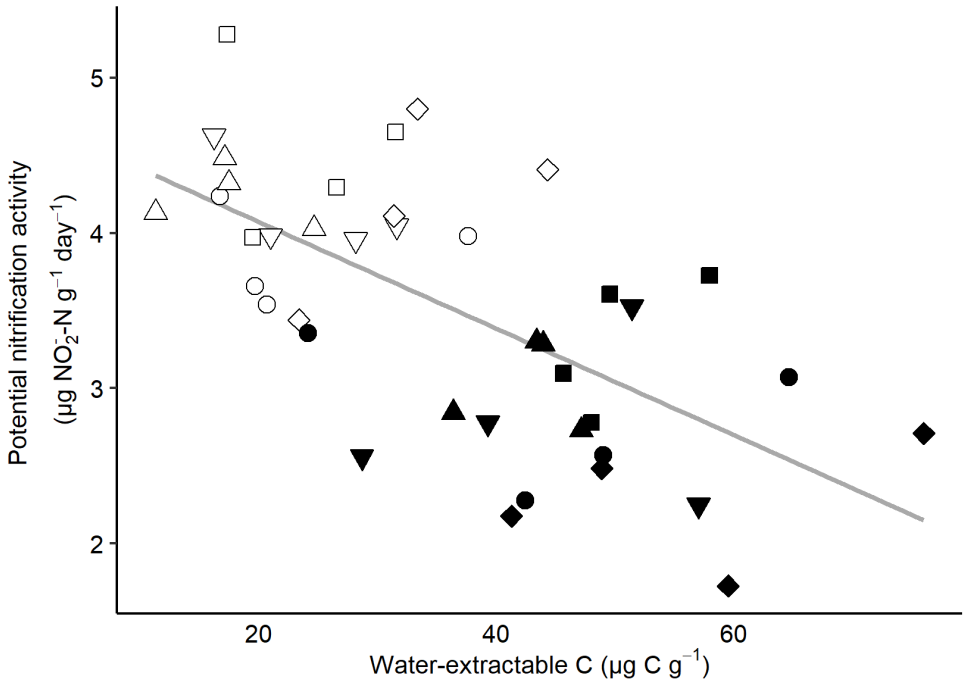

Figure 3.4. Linear relationship between potential nitrification activity (N_p) and water-extractable C concentrations (C_{we}) for *Cichorium intybus* (○), *Lolium perenne* (□), *Plantago lanceolata* (◇), *Raphanus raphanistrum* (△), and *Raphanus sativus* (▽) with low N (white symbols) and high N (black symbols) treatments, n = 40. Values are expressed as per unit dry mass of soil. The linear regression is shown as a solid grey line (RMSE = 0.637 µg NO_2^--N g^{-1} day^{-1}, R^2 = 0.406, P < 0.001).

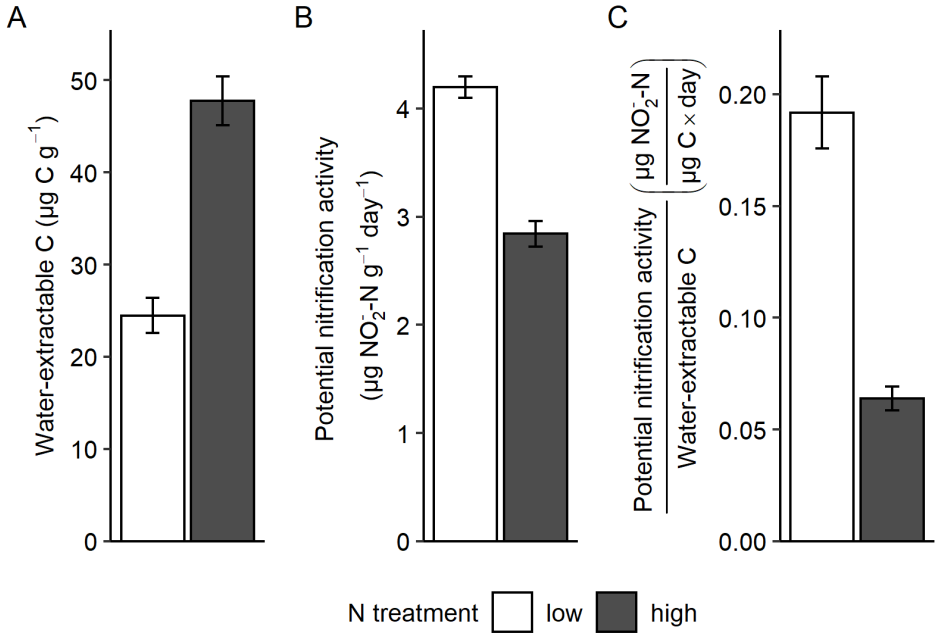

Figure 3.5. Mean water-extractable C concentrations (C_{we}) (A), potential nitrification activity (N_p) (B) and ratio of potential nitrification activity over water-extractable C concentration (C) across all plant species grouped by N treatment. Error bars represent standard errors, n = 20. Values are expressed as per unit dry mass of soil.

3.5 Discussion

3.5.1 Soil nitrification potential and available carbon

The supply of available C has been shown to be an important driver for soil nitrification (Clarholm, 1985; Fisk et al., 2015; Knops et al., 2002; Paterson, 2003). Specifically, sufficient supply of microbially available C can stimulate growth of heterotrophic microbes, leading to increased immobilisation of NH_4^+ and its unavailability to autotrophic nitrifiers (Chen and Stark, 2000; Subbarao et al., 2006). The findings support the assumption of a close coupling of root-derived available C and nitrification activities, showing a negative relationship between C_{we} and N_p across all plant species. However, there were no clear differences between plant species and so the findings do not support the first hypothesis. The overall higher C_{we} in planted than in unplanted soils implies that the C_{we} pool contains significant amounts of rhizodeposits. The correlation between C_{we} and I_C suggests that part of the C_{we} fraction was available for microbial use, supporting the assumption that soluble organic C can be used as an estimate for the available soil C fraction (Chantigny et al., 2014; Embacher et al., 2007; Marschner and Kalbitz, 2003; Pelz et al., 2005). The I_C data were interpreted with caution as the measurements were confounded by differences in water contents of the samples (Parkinson and Coleman, 1991). Nevertheless, the results suggest that available C increased microbial C utilisation as indicated by I_C, which may have stimulated heterotrophic NH_4^+ immobilisation, leading to reduced nitrification activities. Even though soil NH_4^+-N concentrations were marginally higher in the high N than in the low N soils, this NH_4^+ may not be microbially available, since KCl-extractable NH_4^+-N was shown to be a poor indicator for microbially available N (Sawada et al., 2017; Scheu and Parkinson, 1995; Tiunov and Scheu, 1999; Vesterdal, 1998). Therefore, and because soil NH_4^+-N concentrations in all treatments were low, it is possible that the soil microorganisms in the high N soils were limited by NH_4^+. This NH_4^+ limitation would increase for autotrophic nitrifiers if heterotrophic uptake stimulated by root-derived C further removed NH_4^+ by microbial N immobilisation. Since microbial N immobilisation was not measured in this study, the observed decrease in N_p cannot be attributed with certainty to an increase in heterotrophic N immobilisation induced by root-derived available C. However, the results support previous studies that have shown similar relationships between available C and N cycling (Bengtsson et al., 2003; Fisk et al., 2015; Gilliam et al., 2005; Szili-Kovács et al., 2007).

The increases in C_{we} in response to the treatments were likely attributable to higher rhizodeposition rates induced by N addition, because the increase was only apparent in the planted soils with the high N treatment and absent in the unplanted control soils (Henry et al., 2005; Nguyen, 2003, 2003; Warembourg and Estelrich, 2001). Rhizodeposits are the main source of available C to soils (Frank and Groffman, 2009; Pollierer et al., 2007; Sokol et al., 2019), and this was likely enhanced by the increase in shoot biomass resulting from N addition. Where the high N treatment led to an increase in shoot biomass, there was a significant increase in C_{we}, which suggests that increased leaf area, and possibly enhanced photosynthetic activity, led to higher rates of rhizodeposition of available C compounds

(Dilkes et al., 2004; Högberg et al., 2001; Rogers and Humphries, 2000), supporting the second hypothesis. In addition to an overall increase in rhizodeposition, N addition can also contribute to changes in the composition of rhizodeposits (Bowsher et al., 2018). For example, high N availability can result in increasing exudation of amino acids (Carvalhais et al., 2011), which supply soil microorganisms with energetically and metabolically available C and N compounds (Drake et al., 2013).

In addition to rhizodeposition, N inputs may have led to increases in C_{we} by increasing the rate of soil organic matter decomposition, known as 'positive priming' (Conde et al., 2005; Hamer et al., 2009; Kuzyakov et al., 2000). Priming effects are not well understood (Blagodatskaya and Kuzyakov, 2008), with reports of negative or neutral effects with added N inputs to soil (Fornara et al., 2013; Kuzyakov et al., 2001; Ramirez et al., 2012). In this study, the occurrence of positive priming is more likely as C_t concentrations tended to decrease with N addition. Further, lower C_t concentrations were measured in the high N treatments for both the planted and unplanted soils, indicating that the effect cannot be attributed to the presence of roots alone. Similarly, Khalil et al. (2007) measured significant losses in C_t after adding N to unplanted soils. Soil organic matter decomposition in the soil studied here was probably primed by stoichiometrically-regulated interactions between microbial decomposers (R. Chen et al., 2014; Guenet et al., 2010), specific root exudate compounds that chemically disrupt mineral-organic associations (Keiluweit et al., 2015), and a temporary pH increase following urea hydrolysis (Clough et al., 2010; Kelliher et al., 2005; Sherlock and Goh, 1984). However, the lack of a significant difference in both C_{we} and C_t between the high N and low N unplanted control soils suggests that priming effects had a minor influence on C_{we} compared to those from rhizodeposition. Nevertheless, the occurrence of a priming effect cannot be ruled out, and it is likely that soil organic matter decomposition contributed to the observed increase in C_{we}.

3.5.2 Soil nitrification potential and ammonia-oxidising microbial abundance

Nitrifying microbial communities in soils are typically sensitive to environmental changes, such as pH and N supply (Prosser and Nicol, 2012). In this study, the overall greater abundance of AOA relative to AOB in all treatments is probably related to the low pH of the soil, as acidic conditions typically favour AOA over AOB (Prosser and Nicol, 2012). This, and the differences in the response of the AOB and AOA populations to N application, support the current perception of niche differentiation between microbial populations in soils (Martens-Habbena et al., 2009; Prosser and Nicol, 2012). The lack of difference in the abundance of AOB and AOA communities between the planted and unplanted soils supports previous evidence that changes in the soil ammonia-oxidising microbial community are influenced dominantly by the available N supply, while the presence of plants and variation among plant species is less influential (Malchair et al., 2010a, 2010b; Thion et al., 2016). Here, the N addition likely increased AOB biomass growth, as demonstrated previously (Di et al., 2010, 2009; Prosser and Nicol, 2012; Simonin et al., 2015), while an increase in the AOA community has been shown to be largely

independent of soil N concentration, and can occur even with low N supply due to a high affinity for NH_3^+ (Levy-Booth et al., 2014; Martens-Habbena et al., 2009; Schleper and Nicol, 2010).

Ammonia-oxidising microbes are considered to drive soil nitrification (Prosser and Nicol, 2012), and a positive correlations between AOA or AOB abundance and soil NO_3^+-N concentrations or N_p has been shown (Di et al., 2009; Gubry-Rangin et al., 2010; He et al., 2007). Contrary to this, there was a decrease in N_p in the soils with high N addition, together with a marginal increase in soil NH_4^+-N concentration, no difference in AOA abundance, an increase in AOB abundance and little evidence of a direct relationship between AOA or AOB abundance and N_p. As discussed in section *3.5.1*, it was hypothesised that N_p was reduced by strong competition for NH_4^+ between heterotrophic microorganisms and autotrophic nitrifiers, influenced by root-derived available C. Unlike N_p, AOA and AOB gene copy abundance can reflect cumulative effects and antecedent conditions favouring increases in AOA and AOB communities may have occurred earlier and decreased subsequently until the time when destructive sampling took place. Yet, proportions of the AOA and AOB population may have persisted, including dead microbial cells, which would bias the measured microbial community (Carini et al., 2017; Dlott et al., 2015; Levy-Booth et al., 2014). In the context of this study, increased NH_4^+ availability and soil pH in the high N treatments would have likely declined throughout the weeks following the N application (Anderson et al., 2018; Clough et al., 2010; Kelliher et al., 2005) to the levels observed at the time of sampling, which could affect the AOA population and presumably decrease or even degrade the AOB population (Di et al., 2010; Frijlink et al., 1992; Lu and Jia, 2013). Although measurements made at the end of the experiment were not able to capture these dynamics, the measured AOA and AOB abundances most likely included dormant and dead DNA fragments, which would explain the lack of a relationship between AOA or AOB abundance and N_p in both this and previous studies (Hallin et al., 2009; Jordan et al., 2005; Rudisill et al., 2016; Wessén et al., 2010).

3.5.3 Soil nitrification potential and plant species effects

Overall, there was no significant plant species effect on N_p. However, the influence of C_{we} on N_p may have masked the potential effects of plant species. For example, previous studies that have shown a marginal plant species effect on N cycling suggest that other factors dominated N transformations, such as grazing regimes (Le Roux et al., 2003) and soil type (Groffman et al., 1996). Similar to this study, Stienstra et al. (1994) found no significant plant species effect on nitrification activities in a grassland system, but an overall reduction in nitrification when plants were present, which they attributed to enhanced NH_4^+ immobilisation.

The shoot N contents indicated that the plant species were able to take up large amounts of N from the soil, so this may have limited N supply to soil microorganisms. Soil microorganisms are generally more competitive for N uptake than plant roots, but plants benefit from the fast turnover time of microbial biomass that supplies available N (Kuzyakov and Xu, 2013). In this study, soil NH_4^+-N concentrations were low in all treatments but slightly higher in the high N treatments compared to the low N treatments. Although this may suggest that plant roots were not more NH_4^+-limited in the high N soil than in the low N soil, it is possible that this NH_4^+ is bound to clay minerals and thus not easily available for plant and microbial uptake (St. Luce et al., 2011; Vesterdal, 1998). Taken together, it is unlikely that root uptake induced NH_4^+ limitation that led to reduced N_p in the high N treatment.

Another possible explanation for the decrease in N_p in the high N treatments is a potential increase in soil respiration in response to the high N addition (Barnard et al., 2004). Higher respiration would decrease available oxygen, which would limit nitrification (Grundmann et al., 1995). Although no respiration measurements were made, an increase in soil respiration following N addition is possible, as reported in other temperate grassland systems (Craine et al., 2001; S. L. Graham et al., 2014). This is supported by the increased root biomass in the high N treatments, because an increasing root N concentration can be related to enhanced root respiration rates (Bahn et al., 2006).

Even though root N concentration was not measured, the increase in shoot N concentrations for all species in the high N treatments indicates that N was taken up by the plants and thus concentrations may have increased in all plant components. The likely increase in photosynthesis with increased shoot N content could have led to increases in root C concentrations, where the root C:N ratio would remain unchanged. In support of this, Cong and Eriksen (2018) reported that the root C:N ratio of *L. perenne* decreased after the addition of 250 kg N ha^{-1}, while that of *P. lanceolata* remained constant. They related the overall low root C:N ratio of *P. lanceolata* to increased labile C inputs into the soil, which supports the observations of this study of enhanced C_{we} for *P. lanceolata*. However, the lack of significant differences in C_{we} between species within each N treatment does not indicate whether potential differences in root C:N ratios may have affected N_p.

Some studies have observed inhibitory effects on soil nitrification associated with specific plant species, among them *R. raphanistrum* (O'Sullivan et al., 2017) and *P. lanceolata* (Dietz et al., 2013; Luo et al., 2018; Massaccesi et al., 2015). In this study, there were no differences in N_p between any of the species tested. While attempts have been made to relate low nitrification activities to the root-release of biological nitrification inhibitors (BNI) that inhibit nitrification specifically (Carlton et al., 2019; Luo et al., 2018; O'Sullivan et al., 2017), the influence of root-derived available C on NH_4^+ immobilisation has often been overlooked. Although no BNI compounds have yet been identified for any of the plant species used in this study, their possible presence and influence on N_p cannot be excluded. Future research investigating the mechanisms of nitrification inhibition by specific plant species is needed to determine the relative effects of both C and BNI compounds on nitrification.

3.6 Conclusions

The findings provided evidence that the addition of N to grassland plant species increased soil C availability, which is likely attributable to enhanced rhizodeposition. The increased root-derived C was probably available for heterotrophic microbial growth and this may have reduced potential nitrification activity. The findings support growing evidence that the risk of N leaching from soils is greatest under conditions of low available C supply to soil from plants, for example during winter condition or following biomass harvest when photosynthetic activity is low. Maintaining continuous plant cover and active growth is important for increasing plant N uptake and rhizodeposition of available soil C that will lead to increased ecosystem N retention, and reduced N leaching and gaseous losses. Further studies in field conditions are needed to support the development of management practices to increase inputs of available C to soils and reduce N losses.

Chapter 4

High additions of nitrogen affect rhizodeposition and plant species-specific differences in microbial community composition and processing of rhizodeposited carbon

4.1 Abstract

Rhizodeposition by grassland plants is a major process by which carbon (C) is transferred from plants to soil microorganisms. The response of rhizodeposition and microbial processing of rhizodeposited C to grassland management practices has far reaching consequences for the C balance and for soil functional processes that couple C and nitrogen (N) cycling. However, little is known about the effects of high N inputs on C rhizodeposition and processing of rhizodeposited C by soil microbial communities for different grassland plant species. This study investigated the effect of varying N availabilities on plant C balance, C rhizodeposition, microbial processing of rhizodeposited C, and the concomitant regulation of soil functional processes. Measurements of plant C balance and a $^{13}CO_2$ pulse-labelling experiment on grassland microcosms were combined with ^{15}N isotope pool dilution measurements on soil samples. Two common grassland species, *Lolium perenne* (perennial ryegrass) and *Plantago lanceolata* (ribwort plantain) were grown in microcosms under controlled conditions and subjected to four different rates of high N addition (220, 300, 450, and 750 kg N ha^{-1}, respectively) and concurrent clipping.

Overall, photosynthesis, plant C uptake, and C rhizodeposition into the soil were greater for *P. lanceolata* than those for *L. perenne*, leading to plant species-specific differences in the soil microbial community and microbial uptake of rhizodeposited C. Plant C uptake and C rhizodeposition increased for both plant species with increasing N addition, resulting in compositional changes of the microbial community towards a more bacteria-dominated system and enhanced microbial uptake of rhizodeposited C. However, N-induced changes in allocation of rhizodeposited C to different microbial groups were much less pronounced for *P. lanceolata* than that for *L. perenne*, suggesting that microbial processing of rhizodeposited C from different plant species depends on N availability. Although microbial uptake of rhizodeposited C was associated with the rate of soil respiration, there was no significant effect on N mineralisation and nitrification rates, which may suggest a decoupling of soil C and N cycles with increasing N addition. These findings highlight the need to consider plant species responses to high N inputs and the effects of C rhizodeposition on soil microbial communities to interpret the influence of grassland management practices on processes regulating the coupling of soil C and N cycling.

4.2 Introduction

Grassland ecosystems occupy about a quarter of the world's terrestrial surface and provide pivotal ecosystem services (Conant et al., 2017; FAO, 2018; Strömberg, 2011). Increasing grassland intensification and mitigation of the associated negative environmental impacts requires more sustainable grassland management strategies (Foley et al., 2011; Lemaire et al., 2014). In particular, nitrogen (N) inputs from fertilisers and livestock urine are commonly added at high rates on intensively grazed grasslands, leading to large N losses in form of gaseous emissions and N leaching (Cardenas et al., 2010; Dungait et al., 2012a; Galloway et al., 2008). Because soil N and carbon (C) cycles are usually strongly coupled in grassland systems, alterations in C cycling affect the biogeochemical processes related to soil N cycling and their feedback to ecosystem functioning, such as nitrification and soil N retention (Reay et al., 2008; Rumpel et al., 2015; Soussana and Lemaire, 2014). For example, an increasing supply of available C substrates can stimulate microbial N immobilisation by increasing the stoichiometric N demand of the mostly C-limited heterotrophic soil microbial community (Booth et al., 2005; Cleveland and Liptzin, 2007; Soong et al., 2020). Similarly, lab-based studies have shown that manipulating C inputs to improve soil N retention may lead to effective management practices that diminish N losses (Fisk et al., 2015; Vinten et al., 2002). However, the present development of sustainable management practices is constrained by a lack of knowledge about the biochemical and microbial mechanisms that link the C and N cycles in grazed grasslands (Dungait et al., 2012a; Gärdenäs et al., 2011; Macdonald et al., 2018).

Rhizodeposition is a major process by which C is transferred from plants to the soil (Kätterer et al., 2011; Sokol et al., 2019). The amount and composition of rhizodeposits depend, among other factors, on plant species (Jones et al., 2009) and influence the composition of soil microbial communities, resulting in distinct microbiomes for individual plant species (Philippot et al., 2013; Sasse et al., 2018). This root microbiome can provide benefits to the host plant, for example by suppressing pathogens, degrading pollutants, or by supplying available nutrients (Bakker et al., 2018; Berendsen et al., 2012; Bulgarelli et al., 2013; Lugtenberg and Kamilova, 2009). These functions can be affected by changing the amount or type of rhizodeposits, facilitated, for example, by altering the supply of nutrients (Bais et al., 2006; Carvalhais et al., 2013; Ehrenfeld et al., 2005; Haichar et al., 2014; Sasse et al., 2018). However, few studies have explored how variation in rhizodeposition affects the composition and functions of the microbial community (Denef et al., 2009; Haichar et al., 2016).

The processes that couple and decouple soil C and N cycles in high N systems are highly complex and further understanding of their underlying mechanisms is necessary to determine management practices that will reduce C and N losses through uncontrolled decoupling (Rumpel and Chabbi, 2019). In natural ecosystems or systems with low to moderate N inputs, efficient plant N acquisition has been shown to rely on the activity of microbial symbionts (Lambers et al., 2008; Tao et al., 2019). To maintain high activity of these microbial symbionts, plants may invest in rhizodeposits that stimulate microbial activity

(Lambers et al., 2008; Paterson, 2003). In systems with high N inputs, however, microbial uptake of rhizodeposited C can differ. Increased photosynthesis may increase rhizodeposition, which may lead to greater microbial uptake of rhizodeposited C (Nguyen, 2003). Conversely, microbial uptake of rhizodeposited C can decrease, because the host plant's investment in microbial symbionts through rhizodeposits is no longer required for efficient N acquisition (Cavagnaro et al., 2015; Johnson, 2010; Leff et al., 2015). High N inputs may not only shift the uptake of rhizodeposited C by microbial groups, but may also lead to significant compositional changes in the soil microbial community (Denef et al., 2009; Leff et al., 2015). Past studies have shown that N availability altered the soil microbial community composition or microbial uptake of rhizodeposited C, which led to significant changes in microbially-mediated functions related to C and N cycling (Bengtson et al., 2012; Cookson et al., 2007; Högberg et al., 2010; Paterson et al., 2007). However, few studies have considered the effects of high N loading rates that are typical for grazed grassland systems (Denef et al., 2009). Thus, the effect of high N addition on soil functional processes through alterations in rhizodeposition and the soil microbial community is poorly understood.

The aim of this study was to investigate the effects of inorganic N addition on plant C balance, C rhizodeposition and microbial community regulation of soil functional processes related to C and N cycling for two grassland plant species. A $^{13}CO_2$ pulse-labelling approach with two different plant species growing in microcosms in controlled conditions was used to trace photosynthetically fixed ^{13}C through the plant-soil system in combination with short-term measurements of ^{15}N pool dilution to assess gross N transformation rates. This experiment was designed to test three hypotheses: (1) Increasing N addition will increase plant C uptake, C rhizodeposition to soil, and change the microbial community composition. (2) Differences in C rhizodeposition by different plant species will affect the composition of the soil microbial community and microbial uptake of rhizodeposited C. (3) Changes in microbial community composition will affect microbial utilisation of rhizodeposited C, soil respiration rates, and soil N transformation rates. The effects of C rhizodeposition on microbial C and N cycling were investigated for four N addition treatments and for the two plant species, *Lolium perenne* and *Plantago lanceolata*. These plant species were chosen because they are common species in grazed grassland systems and have been recognised previously for their contrasting effects on soil C and N dynamics, including a trend for higher C rhizodeposition by *P. lanceolata* compared to that for *L. perenne* in response to N addition (Chapter 3).

4.3 Materials and Methods

4.3.1 Soil collection

A Templeton silt loam soil (Typic Immature Pallic soil (New Zealand Soil Classification, NZSC, Hewitt, 2010); Udic Haplustept (USDA, Soil Survey Staff, 2014)) was collected from an irrigated perennial ryegrass (*Lolium perenne* L.)/white clover (*Trifolium repens* L.) grassland at Lincoln University Research Dairy Farm at Lincoln, New Zealand (latitude 43.640° S, longitude 172.463° E; 14 m above sea level). The site was not grazed and no fertiliser was applied for three years prior to this study. The soil was sieved (≤ 4 mm) to remove stones and plant residues and the gravimetric water content determined by weighing and drying a fresh sample at 105 °C for 24 h. At the time of sampling, the topsoil (0 to 150 mm) pH ($CaCl_2$) was 5.10 and the organic C concentration was 25 g C kg^{-1}. Further soil characteristics are shown in Table A.2.1 (Appendix A.2).

4.3.2 Experimental design

The PVC cylindrical microcosms (192 mm diameter, 280 mm depth) were filled with 10.5 ± 0.3 kg fresh, homogenised soil at an average bulk density of 1.0 Mg m^{-3}. In the centre of each microcosm, a PVC collar (100 mm diameter, 70 mm height) was placed to a depth of 30 mm for measurements of soil respiration rates. The soil surface within the collar was kept free of plants and the periphery of the microcosms was planted with either *Lolium perenne* L. cv. 'Prospect' (perennial ryegrass) or *Plantago lanceolata* L. cv. 'Tonic' (ribwort plantain) (Figure 4.1). About 30 days after planting the seeds, the four different N treatments were applied in solution. The rates of N supply of 220, 300, 450, and 750 kg N ha^{-1} added as ammonium sulphate ($(NH_4)_2SO_4$) were selected to be similar to loading rates from livestock urine patches (Selbie et al., 2015). There were 32 microcosms in a fully factorial design with four replicates for the two plant species and four N treatments.

Figure 4.1. Schematic diagram of microcosm planted with *P. lanceolata* (left) and photo of planted microcosms in plant growth chamber (right), showing both species (*L. perenne* and *P. lanceolata*).

The microcosms were placed in two plant growth chambers (Fitotron HGC 1514, Weiss Gallenkamp, UK) set to 16 h photoperiod, air temperature 22° C, irradiance (400 to 700 nm) 500 µmol m^{-2} s^{-1} at canopy level, and relative humidity 70%. Every week, the microcosms were interchanged between the plant growth chambers and their locations were re-randomised within each chamber to account for between- and within-chamber variability. During the growth period, the microcosms were watered every other day and the mass of water added was adjusted weekly to maintain gravimetric soil water content at 60 to 70% of water holding capacity. Superphosphate was applied at a rate of 45 kg P ha^{-1} at 20 days after the seeds were sown to ensure sufficient nutrient supply for plant growth. Each week, starting from 25 days after sowing, the aboveground biomass was harvested by cutting to 40 mm above the soil surface, dried, and weighed.

4.3.3 Net ecosystem CO_2 exchange

Net ecosystem CO_2 exchange was estimated on a weekly basis during the last four weeks of the experimental period prior to destructive sampling. Following Moinet et al. (2016), a cylindrical transparent polycarbonate chamber (200 mm diameter, 210 mm height) with a high-density foam seal was placed on top of each microcosm. The infrared gas analyser from a portable photosynthesis system (LI-6400XT, LI-COR Biosciences, Lincoln, NE, USA) was placed in the chamber for measurements of CO_2 partial pressure. To maintain constant atmospheric pressure, the chamber was fitted with an open vent to the atmosphere and a small fan ensured that the air in the chamber was mixed thoroughly.

Net ecosystem exchange (F_N) was determined from measurements of CO_2 concentration at the start when the chamber was placed on the microcosm and again after 70 s under full irradiance. Subsequently, the same measurements were made in darkness by covering the chamber with black cloth to determine ecosystem respiration (R_E). Additionally, soil respiration (R_S) was measured by placing a survey chamber connected to an automated system (LI-8100, LI-COR Biosciences, Lincoln, NE, USA) on the central collar in each microcosm. Net photosynthesis (A) was derived from the difference between R_E and F_N, adopting sign the convention with positive F_N as net uptake of CO_2 by the ecosystem. Plant respiration rate (R_p) was estimated by subtracting R_s from R_E.

4.3.4 $^{13}CO_2$ pulse-labelling and destructive sampling

At seven and eight weeks after the seeds were sown, the plants were pulse-labelled with $^{13}CO_2$ by placing the microcosms in a sealed transparent, acrylic fumigation chamber (2 m width, 1.2 m depth, 1 m height; for details see Carmona et al. (2020)) for 4.5 to 5 h based on the CO_2 uptake rate. The plants were labelled in two batches within one week for logistical reasons.

Highly enriched $^{13}CO_2$ was prepared for injection into the fumigation chamber by adding 0.6 M citric acid to 20 g of ^{13}C-labelled sodium carbonate ($Na_2{}^{13}CO_3$, 99 atom% ^{13}C, Sigma Aldrich). The $^{13}CO_2$ fumigation led to an increase in CO_2 partial pressure from ambient up to 2100 µmol mol^{-1} with 80% comprised of $^{13}CO_2$. The fumigation was ended when the CO_2 partial pressure in the fumigation chamber had had fallen because of photosynthesis by the plants to approximately 280 µmol mol^{-1}.

One day after labelling, the microcosms were sampled destructively for measurements of plant biomass and elemental concentrations, soil chemistry, soil microbial community composition, and the basal rate of soil respiration and gross N transformation rates. All plant and soil samples were stored in sealed bags and refrigerated at 4 °C until further processing unless stated otherwise. Additional soil subsamples were frozen and freeze-dried immediately after destructive sampling.

4.3.5 Plant analyses

Roots were separated from soil by careful sieving and washing with tap water. Shoot and root samples were weighed after drying at 65 °C for 72 h. To determine the allocation of ^{13}C translocated within the plants, subsamples of dried shoots and roots were ground and analysed for total C and N concentrations and $\delta^{13}C$ using an elemental analyser (Sercon GSL, Crewe, UK) attached to a continuous flow isotope-ratio mass spectrometer (IRMS) (Sercon 20-22, Sercon, Crewe, UK).

4.3.6 Soil chemical analyses

Soil pH was determined on a freeze-dried soil sample in 0.01 M $CaCl_2$ (1:2.5 w:v) (Hendershot et al., 2008; Miller and Kissel, 2010). Mineral ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were measured on extracts of fresh soil with 2 M KCl (1:10 w:v) (Rayment and Lyons, 2011) immediately after destructive sampling, analysed by flow injection (FOSS FIAstar 5000, Foss Tecator AB, Hoganas, Sweden). Total organic C (C_t) and total N (N_t) concentrations were measured on freeze-dried soil samples by dry combustion in an elemental analyser (Sercon GSL, Crewe, UK) attached to a continuous flow isotope-ratio mass spectrometer (IRMS) (Sercon 20-22, Sercon, Crewe, UK).

For water-extractable C concentration (C_{we}), a modified method of Ghani et al. (2003) was used on fresh soil samples that had been refrigerated for 3 weeks after destructive sampling. Briefly, 8 g dry soil equivalent was eluted with 40 mL deionised water (1:5 w:v) for 30 min, then centrifuged (3000 × g for 20 min) and filtered (0.45 µm). Cooled conditions (4 °C) were maintained throughout the whole extraction process to reduce microbial activity, which otherwise could have minimised the ^{13}C recovery in the water-extractable C fraction (Chantigny et al., 2014; Rousk and Jones, 2010). The extract was analysed for C_{we} by measuring the C concentration (Shimadzu TOC Analyser model 5000A with ASI-5000A, Shimadzu Oceania Pty Ltd., Sydney, Australia), and a freeze-dried subsample of the extract analysed for the isotopic $\delta^{13}C$ composition in an elemental analyser (Sercon GSL + IRMS Sercon 20-22, Crewe, UK).

4.3.7 Microbial community composition and rhizodeposited C uptake

To characterise the microbial community and its assimilation of rhizodeposited C, compound specific ^{13}C in phospholipid fatty acids (^{13}C-PLFAs) were analysed. The extraction, fractionation, and methylation of PLFAs was performed following Bligh & Dyer (1959) modified by White et al. (1979) and Frostegård et al. (1991), using modified methods from Quideau et al. (2016). Lipids were extracted from 5.0 g freeze-dried soil with a mixture of chloroform, methanol, and citrate buffer (1:2:0.8 v:v:v) and phospholipids isolated by silica-bonded columns. Phospholipids were transesterified to fatty acid methyl esters (FAME) by mild alkaline methanolysis using methanolic KOH. Fatty acid methyl esters and their isotopic $\delta^{13}C$ composition were analysed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) (Delta plus coupled with TraceGC, Thermo Finnigan, Bremen, Germany) fitted with a DB5-DB1 fused silica capillary column combination (both 30.0 m × 0.25 mm i.d. × 0.25 µm, Agilent Technologies) at the Centre for Stable Isotope Research Analysis, Georg August University Göttingen, Germany. All samples were run in splitless mode using 1 µL of sample solution and helium flow rate of 1.2 mL min^{-1}. After sample injection, the oven temperature was held at 80 °C for 1 min, then ramped to 171 °C at 10 °C min^{-1} and to 193 °C at 0.7 °C min^{-1} with 4 min hold, followed by further heating to 196 °C at 0.7 °C min^{-1} and to 210 °C at 1.5 °C min^{-1}. Finally, the temperature was

ramped at 10 °C min^{-1} to 300 °C where it was held for 7 min. Compounds were converted to CO_2 at 940 °C in the combustion unit fitted with a ceramic tube (internal diameter 0.5 mm) containing an oxidised Ni-Pt-Cu wire. A mix of bacterial fatty acid methyl esters (Supelco 47080-U, Sigma-Aldrich) served as a qualitative standard. Two internal standards (C13:0 and C19:0) were used to identify FAMEs and to calculate FAME concentrations. The C19:0 standard was further used for correcting the isotopic $\delta^{13}C$ composition of individual FAMEs for the C atom that was added during transesterification (Abraham et al., 1998; Bahn et al., 2013). The PLFAs i14:0, i15:0, a15:0, i16:0, i17:0, a17:0 were used as indicators for gram-positive bacteria, the PLFAs 16:1ω7, cy17:0, cy19:0 were designated to gram-negative bacteria, and non-specific PLFAs 14:0, 15:0, 16:0, 17:0 and 18:0 were used as general bacterial biomarkers (Waldrop and Firestone, 2004; Zelles, 1999, 1997). The PLFAs 10Me16:0 and 10Me18:0 were used to indicate actinomycetes (Vestal and White, 1989). The PLFA 16:1ω5 was used as an indicator for arbuscular mycorrhizal fungi (AMF) (Olsson, 1999), whereas 18:2ω6,9, 18:3ω6,9,12 and 18:1ω9 were used as general fungal biomarkers (Frostegård and Bååth, 1996; Stahl and Klug, 1996; Vestal and White, 1989). The AMF biomarker 16:1ω5c was interpreted with care, as it was shown to also occur in gram-negative bacteria (Ruess and Chamberlain, 2010). However, because the patterns of $\delta^{13}C$ composition for the PLFA 16:1ω5c varied strongly from those of gram-negative bacterial PLFA biomarkers, the majority of 16:1ω5c probably originated from microbial groups other than gram-negative bacteria.

4.3.8 Basal soil respiration rate

Basal soil respiration rate (R_{basal}) and the isotopic composition of respired CO_2 was measured on fresh soil samples from each microcosm. A soil sample equivalent to 3 g dry mass was placed in a 12 mL glass incubation vial and the headspace flushed with CO_2-free air. After incubating the vial at 25 °C in the dark for 2 h, a gas sample of the headspace air was taken and CO_2 partial pressure and its isotopic $\delta^{13}C$ composition were determined by injecting the sample into a CO_2-free airstream and analysed using tunable diode laser absorption spectroscopy (TGA100A, Campbell Scientific Inc., Logan, UT, USA). R_{basal} was determined from the increase in total CO_2 partial pressure in the headspace between the start and the end of the incubation period. The partial pressure of ^{13}C above ambient was assumed to be derived from recent rhizodeposits (R_{13C}).

4.3.9 Gross N transformation rates

Two days after destructive sampling, microbial N mineralisation (N_{min}) and nitrification (N_{nit}) rates were determined on fresh soil samples by ^{15}N pool-dilution in a paired treatment following Kirkham and Bartholomew (1954) and Murphy et al. (2003), using modified methods from Bengtsson et al. (2003) and Braun et al. (2018). A volume of 400 µL of a tracer solution containing either $(^{15}NH_4)_2SO_4$ (98% $^{15}N_2$; Cambridge Isotope Laboratories, MA, USA) and KNO_3 or $(NH_4)_2SO_4$ and $K^{15}NO_3$ (99% ^{15}N; Cambridge Isotope Laboratories, MA, USA) was added to 7.6 g fresh soil and mixed gently. The concentration of the tracer solution varied according to the initial soil NH_4^+ and NO_3^- concentrations so that the tracer addition increased the respective product pool by no more than 25% but still resulted in a sufficient enrichment with ^{15}N. This approach was used to avoid stimulation of microbial N consumption by the added substrate (Davidson et al., 1991). The simultaneous addition of NH_4^+ and NO_3^- ensured that both samples were treated the same except for the N isotopic composition. The labelled soil samples were incubated in the dark at 22 °C.

The incubation was ended at 3 h and 24 h after tracer addition by extraction with 2 M KCl as described above but with 1:5 w:v ratio. The incubation time interval was shown to be sufficient for the required constancy of N transformation rates in a similar grassland soil (Braun et al., 2018). The extracts were held frozen (-20 °C) until further processing.

The total concentrations of NH_4^+ and NO_3^- in the extracts were measured by flow injection analysis (FOSS FIAstar 5000, Foss Tecator AB, Hoganas, Sweden). The $^{15}N/^{14}N$ isotopic composition in the extracts was measured by continuous flow-IRMS (Sercon 20-22, Sercon, Crewe, UK) after conversion of NH_4^+ and NO_3^- into nitrous oxide (N_2O). Ammonium was oxidised to N_2O with alkaline sodium hypobromide (NaOBr) solution (10 M NaOH matrix) under the presence of a copper (Cu^{2+}) catalyst (Laughlin et al., 1997), while NO_3^- was transformed to N_2O by cadmium reduction at pH 4.7 (Stevens and Laughlin, 1994) after removal of NH_4^+ by microdiffusion (Keeney and Nelson, 1982). Gross N transformation rates were calculated according to Wessel & Tietema (1992).

4.3.10 $\delta^{13}C$ isotopic compositions

The $\delta^{13}C$ isotopic composition was reported as the relative difference between the sample and the standard values (Equation 4.1):

$$\delta^{13}\mathrm{C}\ (‰) = \left(\frac{R_{\mathrm{sample}}}{R_{\mathrm{standard}}} - 1\right) \times 1000 \qquad (4.1)$$

Where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratio of the sample and the Vienna Pee Dee Belemnite (VPDB) international reference standard ($^{13}C/^{12}C = 0.01118$), respectively.

The ^{13}C fractional abundance ($f^{13}C$) in atom% was derived from the $^{13}C/^{12}C$ ratio (Equation 4.2):

$$f^{13}\mathrm{C}_{\mathrm{sample}}(\mathrm{atom\%}) = R_{\mathrm{sample}} \times 100 \tag{4.2}$$

The increase in ^{13}C atoms induced by pulse labelling was calculated as the difference between the enrichment with ^{13}C atoms in the sample relative to that in an unlabelled control and expressed as percentage of total C atoms present (atom% excess, $\mathrm{APE}^{13}\mathrm{C}_{\mathrm{sample}}$) (Equation 4.3):

$$\mathrm{APE}^{13}\mathrm{C}_{\mathrm{sample}}\ (\mathrm{atom\%\ excess}) = \left(f^{13}\mathrm{C}_{\mathrm{sample}} - f^{13}\mathrm{C}_{\mathrm{control}}\right) \times 100 \tag{4.3}$$

Where $f^{13}C_{sample}$ and $f^{13}C_{control}$ are the fractional ^{13}C abundances (atom%) of the sample and the unlabelled control, respectively. The amount of newly assimilated ^{13}C ($^{13}C_{excess}$) that was incorporated into plant or soil pools are expressed on a mass basis, which accounts for the total mass of C present in the respective pool (Studer et al., 2014) (Equation 4.4):

$$^{13}C_{\mathrm{excess}} = \frac{\mathrm{APE}^{13}\mathrm{C}_{\mathrm{sample}}}{100} \times C_{\mathrm{sample}} \tag{4.4}$$

Where C_{sample} is the total C concentration per unit soil or plant dry mass in the respective sample.

4.3.11 Statistical analyses

All data analyses were carried out using R version 4.0.3 (R Core Team, 2020). For all statistical analyses, *P*-values less than 0.05 were considered significant. Values of the parameters from linear mixed-effects models are reported with 95% confidence intervals (95% CI).

Significant enrichment with ^{13}C relative to natural ^{13}C abundance was assessed for each treatment using the one-sample *t*-test. The effects of the plant species (discrete variable with 2 levels) and N treatments (continuous variable) and their interaction on plant, soil, and microbial variables were tested using analysis of variance (ANOVA) for the multiple linear regression models. For testing the treatment effects on net ecosystem CO_2 exchange and its components, repeated-measurement ANOVA for linear mixed-effects models were used with 'measurement day' (3 levels) as a random effect ('lme4' package in R; Bates et al., 2015). Residual plots and plots of predicted vs. observed values were used to verify that the assumptions of normality and homoskedasticity were met. Ratio variables were log-transformed before linear regression to reduce skewness (Gelman and Hill, 2007). To investigate associations between two variables, Spearman's rank correlations were used and correlation coefficients (ρ) reported.

The effects of plant species and N treatment on PLFA-C concentration were tested with a linear mixed effects model ('*lme4*' package in R; Bates et al., 2015) where the intercept and the slope of the PLFA-C concentration were allowed to vary as random effects for individual PLFAs within a given treatment.

The PLFA-C concentrations were log-transformed to meet the assumptions of normality and homoskedasticity of the residuals.

All microbial community abundance data were converted to relative abundances (mol% PLFA g^{-1}) and standardised using Wisconsin Double standardisation procedure prior to analysis using the *'vegan'* package (Oksanen et al., 2019). Permutational multivariate analysis of variance (PERMANOVA) with 'block' as a random effect was used to test the impacts of N addition and plant species on microbial community composition. Multivariate homogeneity of group dispersion was confirmed by following the procedure of Anderson (2006). For visual representation of community data, non-metric multidimensional scaling (NMDS) with Bray-Curtis distances was used (McCune and Grace, 2002). Relationships between the ordinated community composition and soil functional processes were investigated by vector fitting onto the NMDS plot.

4.4 Results

4.4.1 Net ecosystem CO_2 exchange components

Net ecosystem CO_2 exchange (F_N) was 10% higher in the *P. lanceolata* than in the *L. perenne* microcosms (P = 0.005), indicating significantly greater net C uptake over the measurement period (Table A.2.2; Appendix A.2). Although statistically significant (P = 0.002), the effect of N addition was small, with a difference in F_N of -12.7 mg C m^{-2} h^{-1} for each 100 kg N ha^{-1} added for both plant species. About 18% of the variation in the data was explained by the measurement date. Net photosynthesis (A) and soil respiration rate (R_s) was similar across the N treatments for both species (P = 0.066 and P = 0.072, respectively). There were significant differences in A and R_s between the plant species, in that A was overall 16% higher (P < 0.001) while R_s was 10% lower (P = 0.016) for *P. lanceolata* compared to *L. perenne*, respectively. The measurement date explained 0 and 17% of the variation in the data for A and R_s, respectively. Aboveground plant respiration rate (R_p) for *P. lanceolata* was much higher than that for *L. perenne* by a factor of 2.2 (P < 0.001). The increasing rates of N addition were associated with a slight but significant increase in R_p of 16.2 mg C m^{-2} h^{-1} for each 100 kg N ha^{-1} added (P = 0.007). About 9% of the variance in R_p was explained by the measurement date. For visualisation, F_N, A, R_s, and R_p are shown over time in Figure 4.2.

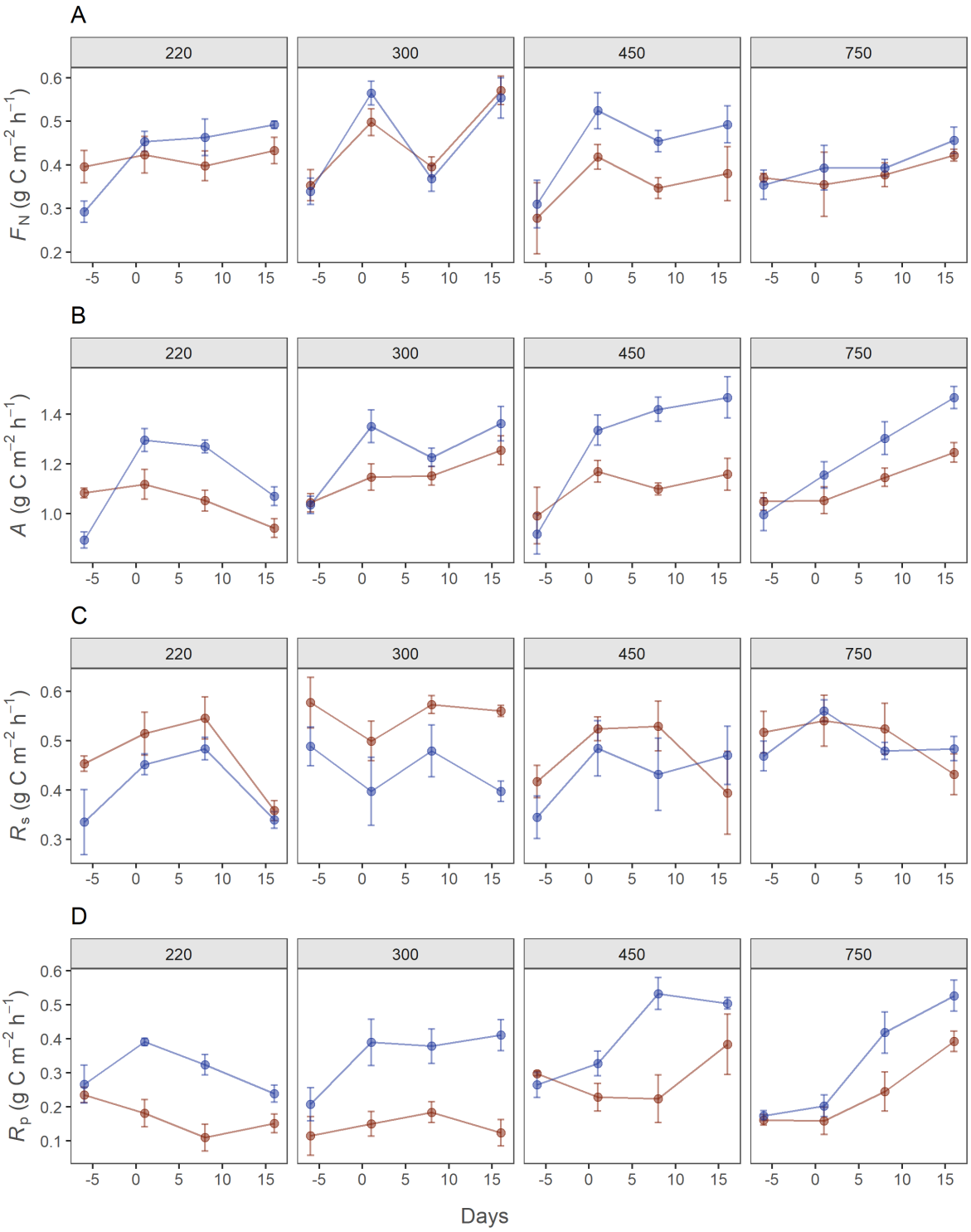

Figure 4.2. Net ecosystem exchange, F_N (A), net photosynthesis, A (B), soil respiration rate, R_s (C), and plant respiration rate, R_p (D) for *L. perenne* (red) and *P. lanceolata* (blue) before and after applying the respective N treatment. Nitrogen treatments were applied on day = 0. Grid-columns represent the different N treatments (kg N ha^{-1}). Depicted are means ± standard errors (n = 4).

4.4.2 Plant biomass, C and N contents, and ^{13}C allocation

Mean shoot biomass increased by 1.12 g per 100 kg N ha^{-1} added and was consistently about 43% higher for *L. perenne* than that for *P. lanceolata* (Figure 4.3A, Table 4.1). Mean root biomass was 13% higher overall for *L. perenne* than that for *P. lanceolata* but decreased by 0.346 g per 100 kg N ha^{-1} added (Figure 4.3B). Overall, mean shoot C and N contents of *L. perenne* were 941 mg C and 60.6 mg N higher than those of *P. lanceolata* and, for both plant species, the values increased with N addition by 4.47 mg C and 1.01 mg N per kg N ha^{-1} added, respectively (Figure 4.3C). However, for root C and N contents, there was a significant interaction between plant species and N addition rate, which was due to a stronger increase in mean root N content and a lower decrease in mean root C content in *P. lanceolata* (slopes of 0.161 mg N and -0.79 mg C per kg N ha^{-1} added, respectively) than for *L. perenne* (slopes of 0.0441 mg N and -2.03 mg C per kg N ha^{-1} added, respectively) as N inputs increased (Figure 4.3D). The mean mass of ^{13}C per unit shoot biomass for *P. lanceolata* was 52% greater overall than that for *L. perenne* (P = 0.005) and mean shoot ^{13}C concentrations increased by 0.294 mg ^{13}C g^{-1} for each 100 kg N ha^{-1} added (P = 0.001; Figure 4.3E). Contrary to this, mean root ^{13}C concentrations were constant across the different rates of N addition (P = 0.812) and were similar between the plant species (P = 0.090; Figure 4.3F). Assuming that shoot N concentration remained constant throughout the experiment, between 61 and 72% (mean = 64%) of N added was taken up by both plant species and partly removed by regular clipping.

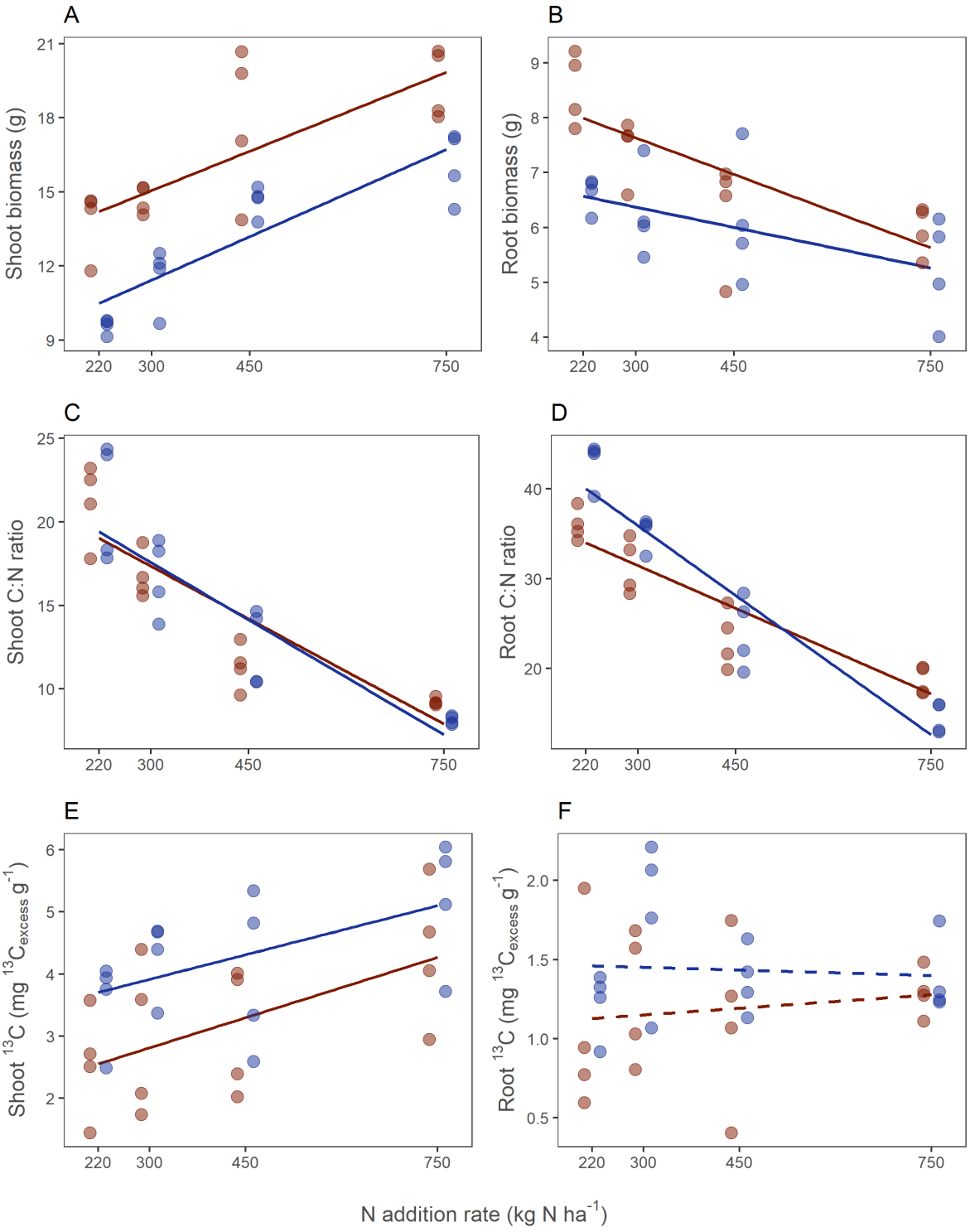

Figure 4.3. Biomass, C:N ratios, and $^{13}C_{excess}$ concentrations of *L. perenne* (red) and *P. lanceolata* (blue) shoots and roots as affected by the different N treatments (kg N ha^{-1}). All treatments were significantly enriched with ^{13}C compared to ambient concentrations ($P < 0.05$). Points were offset on the *x*-axis for visibility. Solid and dashed lines represent significant and non-significant linear relationships for each plant species, respectively.

Table 4.1. Mean and standard errors (in parentheses) of plant variables for each plant species and N treatment (kg N ha^{-1}). *F*-values followed by the respective *P*-values in parentheses and R^2 of regression analysis of plant species and N treatment effects on plant variables are included. NS = not significant ($P > 0.05$).

Response variables (unit)	N addition rate (kg N ha^{-1})				*Species*	N_{rate}	*Species* × N_{rate}	R^2
	220	300	450	750				
Shoot biomass (g)					37.8 (< 0.001)	64.2 (< 0.001)	NS	0.779
L. perenne	13.8 (0.68)	14.7 (0.28)	17.8 (0.53)	19.4 (0.71)				
P. lanceolata	9.57 (0.15)	11.5 (0.64)	14.6 (0.30)	16.1 (0.70)				
Root biomass (g)					11.7 (0.002)	22.8 (< 0.001)	NS	0.543
L. perenne	8.53 (0.33)	7.44 (0.29)	6.30 (0.48)	5.95 (0.23)				
P. lanceolata	6.62 (0.15)	6.25 (0.41)	6.10 (0.58)	5.24 (0.48)				
Shoot C (mg C)					26.3 (< 0.001)	97.1 (< 0.001)	NS	0.810
L. perenne	4172 (230)	4925 (145)	5617 (391)	6902 (380)				
P. lanceolata	3298 (38)	3965 (217)	5068 (59)	5522 (191)				
Shoot N (mg N)					8.7 (0.006)	392.8 (< 0.001)	NS	0.933
L. perenne	199 (14.2)	297 (20.0)	500 (45.0)	748 (36.9)				
P. lanceolata	160 (14.5)	243 (26.4)	419 (41.1)	679 (18.0)				
Root C (mg C)					10.2 (0.004)	29.5 (< 0.001)	5.5 (0.026)	0.589
L. perenne	3208 (139)	2802 (105)	2356 (148)	2058 (130)				
P. lanceolata	2548 (89)	2358 (109)	2376 (219)	2078 (205)				
Root N (mg N)					32.2 (< 0.001)	8.7 (0.006)	31.0 (< 0.001)	0.821
L. perenne	89.6 (5.9)	90.0 (6.3)	101.9 (7.5)	111.6 (11.4)				
P. lanceolata	59.4 (0.5)	67.0 (2.1)	98.7 (3.0)	142.6 (7.2)				

4.4.3 Soil properties and ^{13}C concentrations

Mean soil pH decreased significantly by 0.066 units per 100 kg N ha^{-1} added and was overall approximately 0.1 units lower for *P. lanceolata* than that for *L. perenne* (Table 4.2). There was no statistically significant change in mean total organic C concentration (C_t) associated with increasing rate of N addition or between the plant species. In contrast, mean total soil N concentration (N_t) increased by 15.4 mg N kg^{-1} per 100 kg N ha^{-1} added. Mean soil NH_4^+-N and NO_3^--N concentrations increased exponentially with N addition with proportional gains of 75 and 58% per 100 kg N ha^{-1} added, respectively, whereas plant species had no significant effect. Mean water-extractable C concentration (C_{we}) was similar between the plant species and N addition treatments. Overall, mean total organic ^{13}C concentrations ($^{13}C_t$) for *P. lanceolata* were about double of those for *L. perenne* (Figure 4.4A) and there was no statistically significant change with increasing N addition. In contrast, mean water-extractable ^{13}C concentration ($^{13}C_{we}$) increased significantly by approximately 30% per 100 kg N ha^{-1} added and values were in general about 62% higher for *P. lanceolata* than those for *L. perenne* (Figure 4.4B).

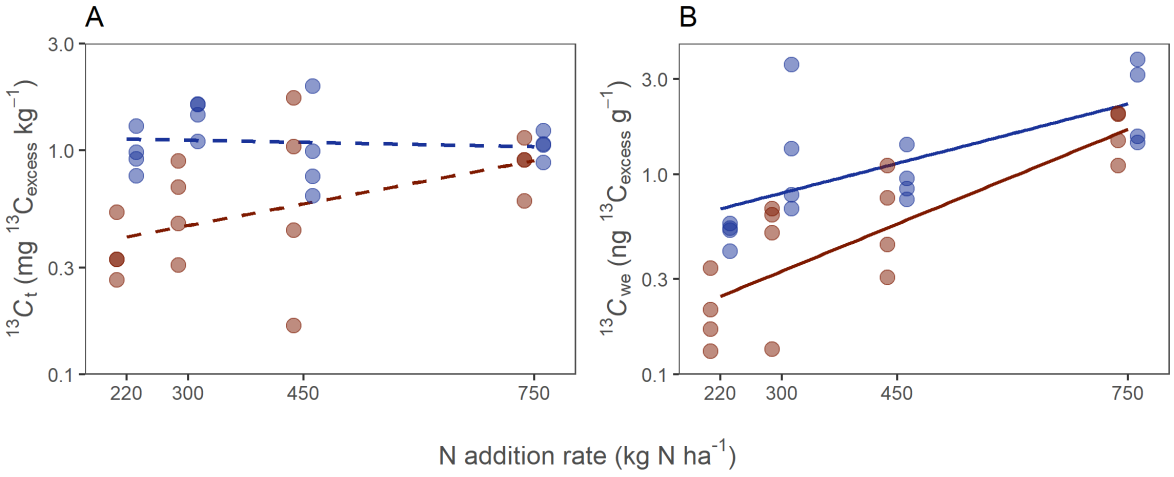

Figure 4.4. Concentrations of total organic ^{13}C, $^{13}C_t$ (A), and water-extractable ^{13}C, $^{13}C_{we}$ (B) under *L. perenne* (red) and *P. lanceolata* (blue) as affected by the N treatments. All treatments were significantly enriched with ^{13}C compared to ambient concentrations ($P < 0.05$). Points were offset on the *x*-axis for visibility. Solid and dashed lines represent significant and non-significant linear relationships for each plant species, respectively. $^{13}C_t$ and $^{13}C_{we}$ are plotted on a log-scale.

Table 4.2. Mean and standard errors (in parentheses) of soil variables for each plant species and N treatment (kg N ha^{-1}). *F*-values followed by the respective *P*-values in parentheses and R^2 of regression analysis of plant species and N treatment effects on soil variables are included. NS = not significant ($P > 0.05$).

Response variables (unit)	N addition rate (kg N ha^{-1})				*Species*	N_{rate}	*Species* × N_{rate}	R^2
	220	300	450	750				
pH					35.2 (< 0.001)	299.0 (< 0.001)	NS	0.920
L. perenne	4.89 (0.01)	4.79 (0.01)	4.68 (0.02)	4.53 (0.03)				
P. lanceolata	4.80 (0.01)	4.70 (0.01)	4.58 (0.02)	4.43 (0.03)				
C_t (g C kg^{-1})					NS	NS	NS	NS
L. perenne	22.3 (0.3)	22.4 (0.2)	22.5 (0.4)	22.2 (0.2)				
P. lanceolata	22.8 (0.1)	22.4 (0.2)	21.8 (0.2)	22.7 (0.3)				
N_t (g N kg^{-1})					NS	15.6 (< 0.001)	NS	0.352
L. perenne	1.89 (0.03)	1.87 (0.01)	1.88 (0.02)	1.94 (0.01)				
P. lanceolata	1.87 (0.02)	1.87 (0.02)	1.86 (0.03)	1.97 (0.01)				
NH_4^+-N (mg N kg^{-1})*					NS	352.6 (< 0.001)	NS	0.924
L. perenne	1.82 (0.17)	2.02 (0.25)	9.37 (3.35)	79.66 (14.41)				
P. lanceolata	1.75 (0.28)	2.12 (0.10)	7.75 (2.58)	85.91 (15.62)				
NO_3^--N (mg N kg^{-1})*					NS	34.7 (< 0.001)	NS	0.545
L. perenne	0.21 (0.15)	0.52 (0.27)	1.14 (0.42)	3.31 (0.48)				
P. lanceolata	0.23 (0.17)	0.53 (0.25)	1.18 (0.80)	2.62 (0.67)				
C_{we} (µg C g^{-1})					NS	NS	NS	NS
L. perenne	10.8 (0.39)	9.7 (0.94)	10.4 (0.84)	11.9 (0.61)				
P. lanceolata	11.7 (0.20)	10.4 (0.57)	10.7 (0.41)	11.8 (0.74)				

* log-transformed

4.4.4 Microbial biomass, microbial ^{13}C uptake, and soil functional processes

Plant species and N addition treatments had no statistically significant effect on mean microbial biomass-C concentration (C_{MB}) (Figure 4.5A, Table 4.3). However, mean microbial biomass-^{13}C concentration ($^{13}C_{MB}$) was overall 45% higher for *P. lanceolata* compared to *L. perenne* and increased at 7.4% for each 100 kg N ha^{-1} added for both species (Figure 4.5B). A strong positive correlation between $^{13}C_{MB}$ and $^{13}C_{we}$ demonstrates a concomitant increase for both ^{13}C concentrations (ρ = 0.706; P < 0.001; Figure 4.6A).

While the mean basal soil respiration rate (R_{basal}) was not significantly affected by N addition, there was a significant difference between plant species, where R_{basal} was 21% higher for *P. lanceolata* compared to that for *L. perenne* (Table 4.3). This effect was not evident for basal soil ^{13}C respiration rate (R_{13C}), which did not significantly differ between plant species or with N addition rates. However, there was a significant positive correlation between $^{13}C_{MB}$ and R_{13C} (ρ = 0.517, P = 0.003; Figure 4.6B).

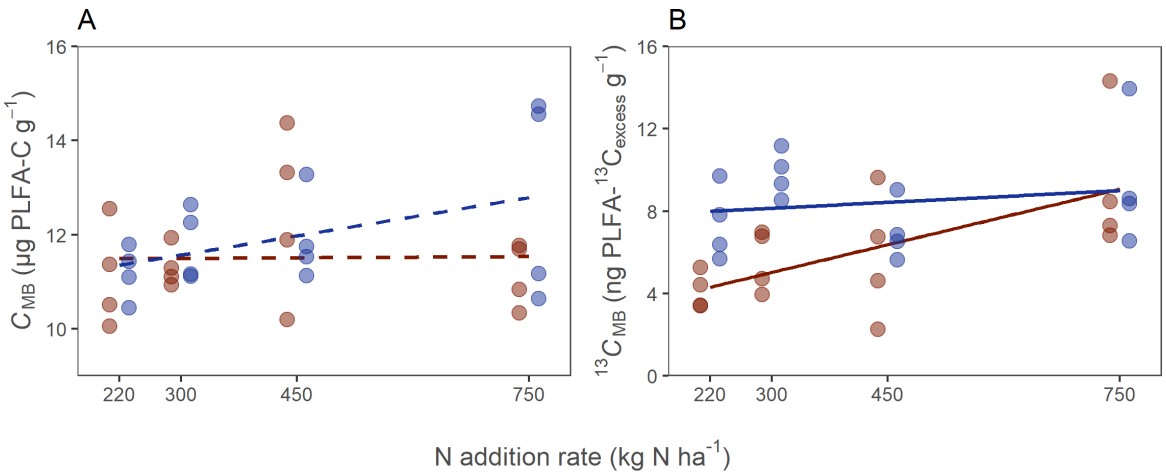

Figure 4.5. Concentrations of soil microbial biomass (µg PLFA-C g soil^{-1}) (A) and soil microbial biomass ^{13}C above ambient (ng PLFA-$^{13}C_{excess}$ g soil^{-1}) (B) under *L. perenne* (red) and *P. lanceolata* (blue) as affected by the N treatments (kg N ha^{-1}). $^{13}C_{MB}$ was significantly enriched with ^{13}C compared to ambient concentrations (P < 0.05). Points were offset on the x-axis for visibility. Solid and dashed lines represent significant and non-significant linear relationships for each plant species, respectively.

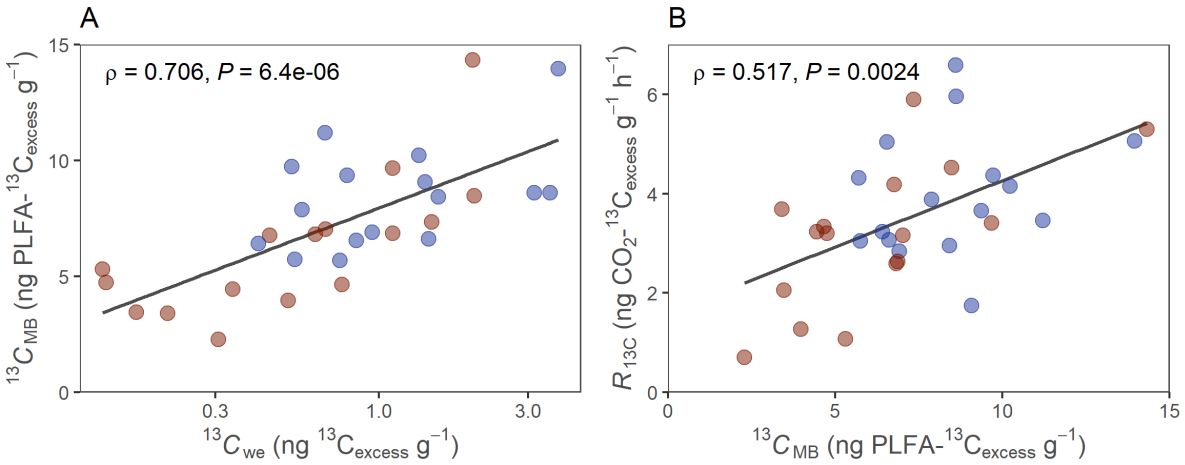

Figure 4.6. Correlation between microbial biomass ^{13}C concentration, $^{13}C_{MB}$, and water-extractable ^{13}C concentration, $^{13}C_{we}$ (A), and basal ^{13}C soil respiration rate, R_{13C} (B) for *L. perenne* (red) and *P. lanceolata* (blue). Grey lines represent significant linear correlations. Correlation coefficients (ρ) and the respective *P*-values were added. $^{13}C_{we}$ are plotted on a log-scale.

Mean gross N mineralization rates (N_{min}) ranged between 3.02 and 5.86 µg N g^{-1} d^{-1} and there was no significant treatment effect (Table 4.3). In contrast, mean gross nitrification rates (N_{nit}) increased significantly with increasing N addition at 31% for each 100 kg N ha^{-1} added, ranging from 0.69 µg N g^{-1} d^{-1} in the lowest N addition treatment to 4.55 µg N g^{-1} d^{-1} in the highest N addition treatment. This increase in N_{nit} was consistent for both plant species. The soil NH_4^+-N concentration correlated significantly with N_{min} (ρ = -0.449; P = 0.010) and N_{nit} (ρ = 0.806; P < 0.001), respectively, but neither N_{min} nor N_{nit} correlated with $^{13}C_{we}$, C_{MB}, or $^{13}C_{MB}$.

Table 4.3. Mean and standard errors (in parentheses) of microbial variables for each plant species and N treatment (kg N ha^{-1}). *F*-values followed by the respective *P*-values in parentheses and R^2 of regression analysis of plant species and N treatment effects on microbial variables are included. NS = not significant ($P > 0.05$).

Response variables (unit)	N addition rate (kg N ha^{-1})				*Species*	N_{rate}	*Species* × N_{rate}	R^2
	220	300	450	750				
C_{MB} (µg PLFA-C g^{-1})*					NS	NS	NS	NS
L. perenne	11.5 (0.6)	11.7 (0.2)	12.8 (0.9)	11.5 (0.4)				
P. lanceolata	11.5 (0.3)	12.2 (0.4)	12.3 (0.5)	13.2 (1.1)				
Fungal:Bacterial ratio*					NS	4.47 (0.037)	NS	NS
L. perenne	0.228 (0.010)	0.217 (0.013)	0.182 (0.023)	0.210 (0.005)				
P. lanceolata	0.213 (0.009)	0.228 (0.013)	0.192 (0.010)	0.177 (0.020)				
Gram+:Gram- ratio*					NS	NS	NS	NS
L. perenne	2.80 (0.15)	2.83 (0.21)	3.40 (0.40)	2.43 (0.12)				
P. lanceolata	2.85 (0.08)	2.69 (0.04)	3.08 (0.26)	3.26 (0.38)				
R_{basal} (µg CO_2-C g^{-1} h^{-1})					20.8 (< 0.001)	NS	NS	0.425
L. perenne	0.518 (0.028)	0.495 (0.016)	0.499 (0.012)	0.552 (0.054)				
P. lanceolata	0.636 (0.031)	0.624 (0.038)	0.586 (0.030)	0.635 (0.042)				
N_{min} (µg N g^{-1} d^{-1})*					NS	NS	NS	NS
L. perenne	5.08 (0.94)	5.86 (1.27)	3.81 (0.57)	3.47 (0.33)				
P. lanceolata	4.66 (1.87)	5.34 (1.17)	3.82 (0.36)	3.02 (0.78)				
N_{nit} (µg N g^{-1} d^{-1})*					NS	66.6 (< 0.001)	NS	0.705
L. perenne	0.83 (0.15)	1.07 (0.11)	2.99 (0.59)	3.34 (0.34)				
P. lanceolata	0.69 (0.06)	0.89 (0.01)	2.70 (0.95)	4.55 (0.88)				

* log-transformed

4.4.5 Microbial community composition and ^{13}C uptake by microbial groups

The microbial community composition differed significantly between the plant species and varied with increasing rate of N addition (Figure A.2.1; Appendix A.2). PERMANOVA indicated that about 13.7% of the variation in the PLFA community composition was explained by the N treatment (P = 0.001), while 11.5% was attributed to the plant species (P = 0.006). Individual PLFA responded distinctly to the plant species and N treatments, with an overall increasing trend in bacterial abundance and decreasing trend in fungal abundance with increasing N addition (Figure A.2.2; Appendix A.2). This led to a slight but significant decrease at 0.03% in the fungal:bacterial ratio for each 100 kg N ha^{-1} added (Figure 4.7A, Table 4.3), whereas there was no significant effect on the gram-positive:gram-negative bacterial ratio (Figure 4.7B). Neither ratio was significantly influenced by the plant species.

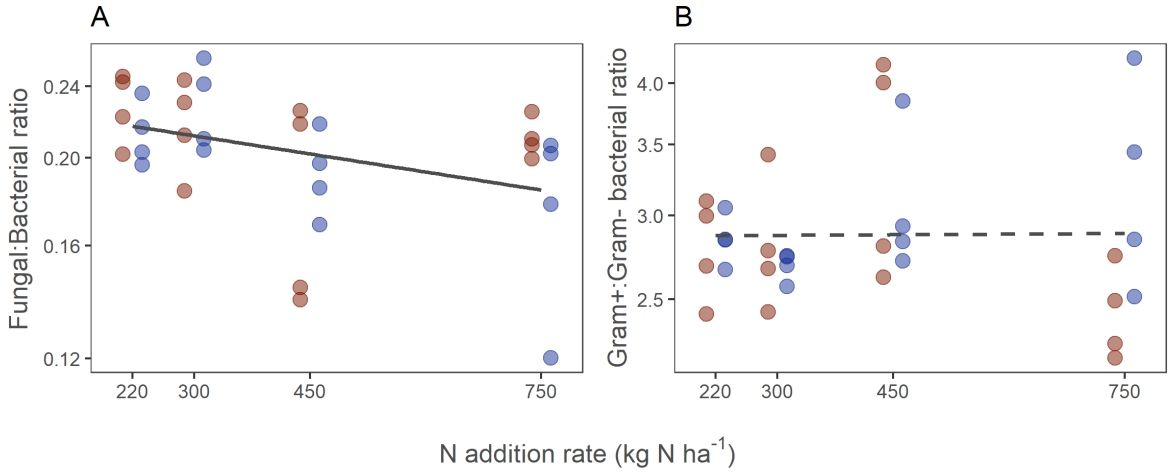

Figure 4.7. Fungal:bacterial PLFA ratio (A) and Gram+:Gram- bacterial PLFA ratio (B) for *L. perenne* (red) and *P. lanceolata* (blue) as affected by the N treatments (kg N ha^{-1}). Points were offset on the *x*-axis for visibility. Solid and dashed lines represent significant and non-significant linear relationships, respectively. Both microbial ratios are plotted on a log-scale.

There were significant linear relationships between the NMDS ordinated microbial community composition and R_{basal} (r^2 = 0.272; P = 0.010) and R_{13C} (r^2 = 0.217; P = 0.027), while relationships with N_{min} and N_{nit} were not significant (P = 0.074 and P = 0.123, respectively; Figure 4.8). The strength and direction of the linear relationships of R_{basal} and R_{13C} with the ordinated microbial community composition appears similar because of the collinearity between the two respiration rates.

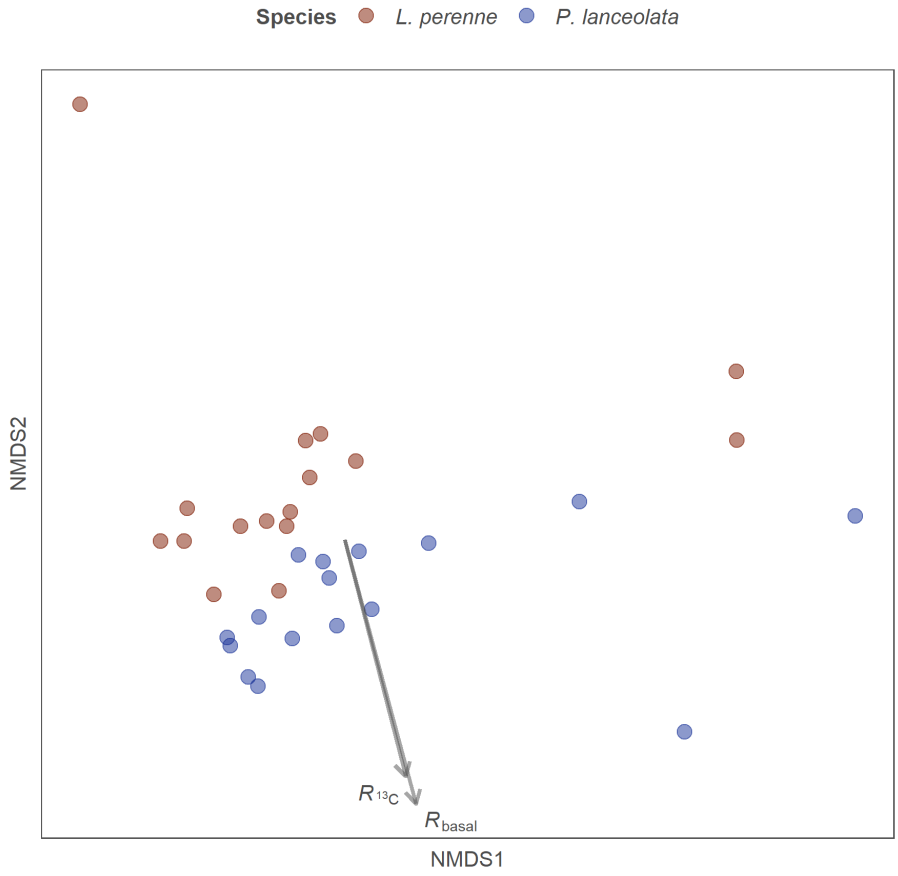

Figure 4.8. Non-metric multidimensional scaling ordination of soil microbial PLFA concentrations (based on mol% PLFA g^{-1}).

Rhizodeposited ^{13}C incorporation was distinct between microbial groups and differed significantly between plant species and with N addition (Figure 4.9). Across all microbial groups, the slope of ^{13}C incorporation with increasing N supply was greater for *L. perenne* than that for *P. lanceolata*, indicating that the soil microbial community under *L. perenne* was much more strongly affected by N addition than that of *P. lanceolata*. In the cases of gram-positive bacteria, ^{13}C incorporation declined with N addition by 1.94% per 100 kg N ha^{-1} added ($P = 0.035$) for *P. lanceolata*, while it increased by 14.2% per 100 kg N ha^{-1} added ($P = 0.010$) for *L. perenne* (Figures 4.9A, B). Similarly, ^{13}C incorporation by gram-negative bacteria decreased for *P. lanceolata* at 9.29% per 100 kg N ha^{-1} added ($P < 0.001$) and increased for *L. perenne* at 22.2% per 100 kg N ha^{-1} added ($P < 0.001$). Rhizodeposited ^{13}C incorporation by fungi increased by 6.37% per 100 kg N ha^{-1} added ($P = 0.032$) for both plant species and was overall about 40.3% higher for *P. lanceolata* than that for *L. perenne* (Figure 4.9C). For AMF, rhizodeposited ^{13}C uptake decreased for both plant species with N addition with proportional declines (per 100 kg N ha^{-1}) of 42% for *L. perenne* ($P < 0.001$) and of 3.3% for *P. lanceolata* ($P = 0.008$) (Figure 4.9D). Actinomycetes were the only microbial group that were not significantly affected by the different N treatments ($P = 0.387$) and the overall ^{13}C incorporation by actinomycetes was approximately 53.9% higher for *P. lanceolata* than that for *L. perenne* ($P = 0.050$; Figure 4.9E).

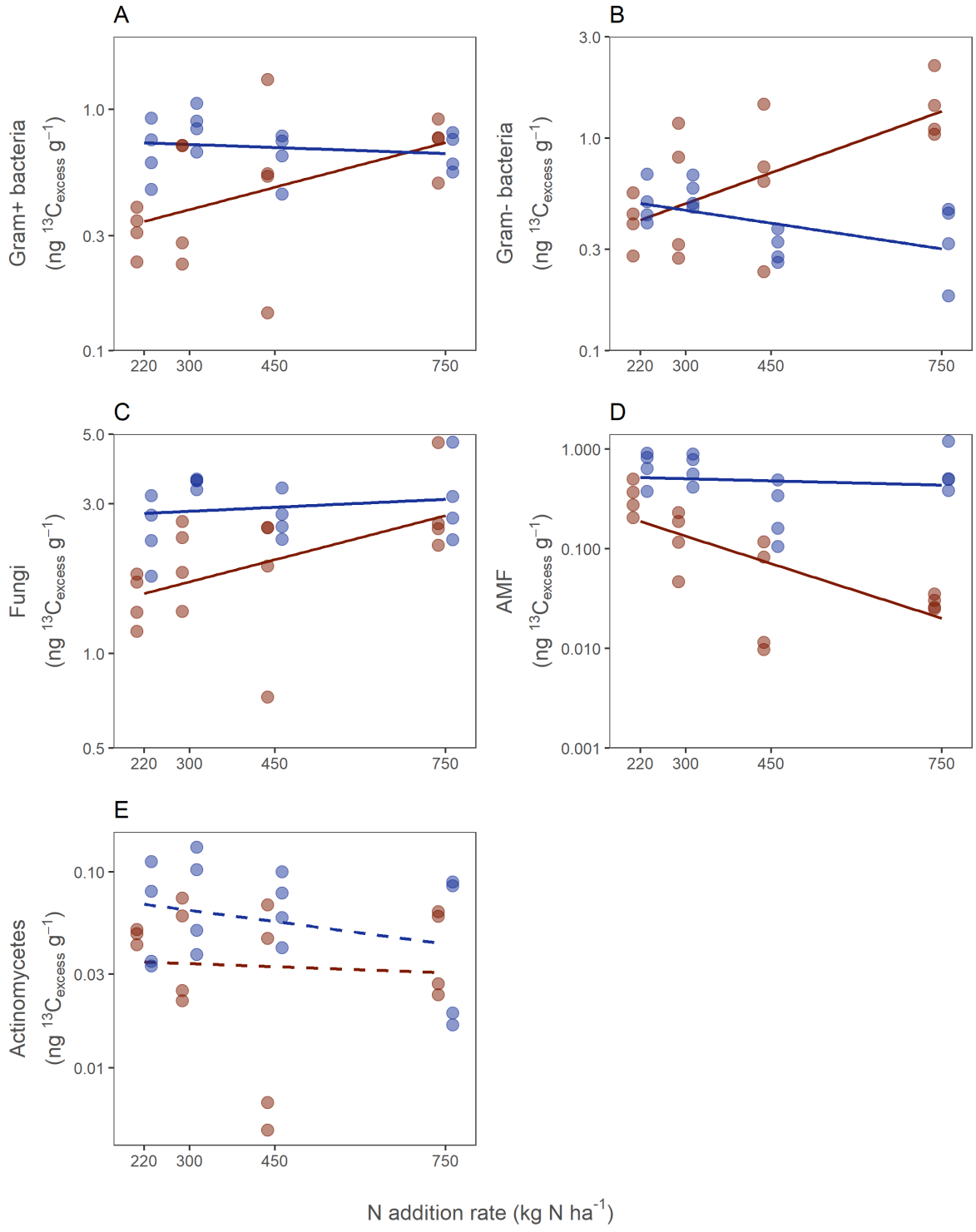

Figure 4.9. Phospholipid fatty acid-^{13}C (PLFA-^{13}C) concentrations of Gram-positive bacteria (A), Gram-negative bacteria (B), Fungi (C), Arbuscular Mycorrhizal Fungi (AMF) (D), and Actinomycetes (E) for *L. perenne* (red) and *P. lanceolata* (blue) as affected by the N treatments (kg N ha^{-1}). All treatments were significantly enriched with ^{13}C compared to ambient concentrations ($P < 0.05$). Points were offset on the *x*-axis for visibility. Solid and dashed lines represent significant and non-significant linear relationships for each plant species, respectively. PLFA-^{13}C concentrations for all microbial groups are plotted on a log-scale.

4.5 Discussion

4.5.1 Nitrogen addition effects

The findings show that plant ^{13}C uptake and ^{13}C rhizodeposition increased with increasing N addition and this resulted in enhanced microbial uptake of rhizodeposited ^{13}C, thus supporting the first hypothesis. Furthermore, increasing N addition led to significant changes in the composition of the soil microbial community.

Increasing N availability enhanced net primary production and plant ^{13}C uptake significantly, leading to an increased release of soluble ^{13}C rhizodeposits. Because plant biomass ^{13}C concentrations were expressed as per unit mass, which normalises ^{13}C concentrations for biomass, the results indicate that plant ^{13}C uptake increased independently from shoot biomass with increasing N addition rates. These observations are consistent with previous studies showing that N addition leads to increased shoot biomass production or leaf surface area and photosynthesis per unit leaf area (Moinet et al., 2016; Niu et al., 2010; van der Werf and Nagel, 1996), consistent with Rubisco activity being closely related to leaf N concentration (Friend, 1991; Hikosaka, 2004). High photosynthesis has been associated with high C translocation belowground into root biomass and rhizodeposition into the soil (Bahn et al., 2009; Giesler et al., 2007; Högberg et al., 2001; Kuzyakov and Cheng, 2001; Pausch and Kuzyakov, 2018; Tang et al., 2005). In this study, there was a distinct ^{13}C enrichment in both root biomass and $^{13}C_{we}$ after 24 h, indicating rapid belowground translocation of recent photo-assimilated ^{13}C, consistent with other studies showing ^{13}C allocation belowground reaching maximum values within 24 to 48 h after labelling in grassland systems (Bahn et al., 2013; De Deyn et al., 2011; Johnson et al., 2002; Leake et al., 2006; Staddon et al., 2014).

The increase in $^{13}C_{we}$ and the unchanged root biomass ^{13}C concentration with increasing N supply may indicate that, as N supply increased, a greater proportion of recent photo-assimilated ^{13}C was released as soluble rhizodeposits rather than invested in root biomass. The decline in root biomass with increasing N supply further supports this and agrees with the 'functional equilibrium theory' that plants well supplied with nutrients invest less in root development (Brouwer, 1963; Poorter et al., 2012). Instead, enhanced plant CO_2 and N uptake in conditions of high N supply may have increased C rhizodeposition into the soil (Bowsher et al., 2018; Nguyen, 2003), which could explain the increase in $^{13}C_{we}$ with increasing N inputs in this study.

There were significant differences in the microbial community composition associated with increasing N addition, with a slight but significant decrease in the fungal:bacterial ratio. These results agree with many other studies reporting that the proportion of bacterial to fungal biomass increases with high N availability (Bardgett et al., 1999; Bradley et al., 2006; de Vries et al., 2006; Leff et al., 2015; Nunan et al., 2006; Paterson et al., 2007; Strickland and Rousk, 2010; Waring et al., 2013; Zhou et al., 2017) and may be related to the higher N demand and uptake capacity of bacteria relative to that of fungi (Myrold

and Posavatz, 2007; Zechmeister-Boltenstern et al., 2015). Conversely, it is possible that the observed differences in the soil microbial community were not induced by N addition per se, but rather by indirect effects, for example changes in rhizodeposition or soil pH (Berg and Smalla, 2009; Bradley et al., 2006; Geisseler and Scow, 2014). Since even small changes in soil pH can significantly alter the soil microbial community (Geisseler and Scow, 2014), the significant decrease in soil pH with increasing N addition observed here might have contributed to the change in the microbial community.

4.5.2 Plant species effects

The findings support the second hypothesis that *L. perenne* and *P. lanceolata* differ in the quantity of ^{13}C rhizodeposition and this influences the soil microbial community composition and microbial uptake of rhizodeposited ^{13}C. Using the mass of ^{13}C recovered in shoot biomass in addition to $^{13}C_{we}$ as a proxy for ^{13}C rhizodeposition within 24 h after labelling, the results suggest that plant CO_2 uptake and C rhizodeposition into the soil were greater for *P. lanceolata* than those for *L. perenne*. This was further supported by the higher photosynthesis, *A*, for *P. lanceolata* relative to *L. perenne*. As mentioned in section *4.5.1*, photosynthesis can increase in response to increased N uptake (Friend, 1991; Hikosaka, 2004). Although, the strength of this relationship between N uptake and photosynthesis depends on species (Hikosaka, 2004) and can vary between grasses and forbs (You et al., 2017), leaf N concentrations were shown to correlate strongly with photosynthesis across a large range of different species (Wright et al., 2004). In the context of this study, relative N acquisition by *P. lanceolata* roots surpassed that of *L. perenne* roots, which likely explains the greater ^{13}C uptake by the forb *P. lanceolata* compared to the grass *L. perenne*. Species characterised by high rates of photosynthesis and N uptake have been associated with greater rates of C rhizodeposition (Henneron et al., 2020; Kaštovská et al., 2017), supporting the greater ^{13}C rhizodeposition by *P. lanceolata* compared to that of *L. perenne* in this study.

The significantly greater $^{13}C_{MB}$ for *P. lanceolata* compared to that for *L. perenne* suggests that the increased quantity of ^{13}C rhizodeposits from *P. lanceolata* stimulated the ^{13}C uptake by soil microorganisms. This was supported by the strong correlation between $^{13}C_{we}$, which is considered to be comprised dominantly of soluble rhizodeposited ^{13}C compounds (Gunina and Kuzyakov, 2015; Hütsch et al., 2002), and $^{13}C_{MB}$, showing that microbial ^{13}C incorporation increased with greater supply of rhizodeposited ^{13}C. Previous studies have demonstrated that labile C compounds from recent rhizodeposits are typically available to soil microorganisms (Pausch and Kuzyakov, 2018). Although soil microorganisms can satisfy their C demand by decomposing complex SOM components, this effect occurs primarily in nutrient-limited environments where microbes mine for the limiting resource and thereby mineralise C (Dijkstra et al., 2013). In the nutrient-rich conditions of this study, however, there was little necessity for the soil microbial community to invest energy in nutrient mining. Therefore, it is likely that rhizodeposited C was the primary source of C substrate for the microbial community.

Consistent with previous studies (Berg and Smalla, 2009; Ehrenfeld et al., 2005; Sasse et al., 2018), both plant species studied here fostered a distinct soil microbial community. Plant species-specific variability in soil microbial community composition has been attributed to net primary productivity (Celestina et al., 2019; Zak et al., 2003), the quantity and quality of rhizodeposits (Berg and Smalla, 2009; Garbeva et al., 2008; Sasse et al., 2018), and soil chemical properties modified by plant species, such as soil pH (Burns et al., 2015). In this study, the soil pH was slightly but significantly lower for *P. lanceolata* compared to that for *L. perenne*, which may have affected the composition of the soil microbial community. As discussed above, our results suggest that ^{13}C rhizodeposition was greater for *P. lanceolata* than that for *L. perenne*, which may have contributed to the dissimilarity in the soil microbial communities. This is further supported by the findings of Ladygina and Hedlund (2010), who related significant differences in the soil microbial communities between *P. lanceolata* and the grass species *Holcus lanatus* to the variation in rhizodeposition.

4.5.3 Relationships among soil microbial communities, rhizodeposited C uptake, and soil functional processes

Supporting the third hypothesis, there was a clear relationship between the soil microbial community composition and microbial uptake of rhizodeposited C. However, the hypothesised effect of the soil microbial community composition and microbial uptake of rhizodeposited C on soil functional processes was only partially supported by the findings. There was no clear evidence for an effect on N_{min} and N_{nit}, whereas R_{basal} and R_{13C} were influenced by changes in the microbial community composition and the uptake of rhizodeposited C.

Nitrogen-induced changes in rhizodeposited ^{13}C uptake by different microbial groups were much less pronounced for *P. lanceolata* compared to *L. perenne*, although the sensitivity of $^{13}C_{we}$ to increased N addition was similar for both species. This was likely related to the difference in the microbial community composition between the species, where, for increasing N addition, *P. lanceolata* preferentially allocated rhizodeposited ^{13}C to AMF and saprotrophic fungi, whereas *L. perenne* allocated rhizodeposited ^{13}C rather to gram-negative bacteria. While past studies have demonstrated that different plant species allocate different quantities of rhizodeposited ^{13}C to various microbial groups (Ladygina and Hedlund, 2010; Ngosong et al., 2011), this study shows that plant species-specific differences in rhizodeposited ^{13}C uptake by microbial groups can be modified by increasing N addition. These findings may have important implications for predictions on C availability and cycling (Fanin et al., 2019; Malik et al., 2016; Six et al., 2006; Wardle, 2004), as the fate of rhizodeposited C from different plant species can change with the soil N supply.

Consistent with previous studies (Gunina and Kuzyakov, 2015; Hütsch et al., 2002; Werth and Kuzyakov, 2008), the correlation between $^{13}C_{MB}$ and R_{13C} suggests that soil microorganisms utilise rhizodeposited ^{13}C compounds for both anabolic cell synthesis and for catabolic respiratory reactions. The proportion of rhizodeposited C used for microbial catabolic and anabolic processes is partially determined by the composition of the microbial community (Soares and Rousk, 2019), which may explain the significant relationship between the ordinated microbial community and R_{13C} in this study.

There was no significant relationship between N_{min} or N_{nit} and $^{13}C_{we}$, $C_{MB,}$ $^{13}C_{MB}$, or microbial community composition, suggesting that neither rhizodeposited ^{13}C accumulation in the soil, nor microbial processing of rhizodeposited ^{13}C influenced N cycling in this study. Availability of rhizodeposited C compounds has been suggested previously to affect soil N cycling by stimulating heterotrophic N immobilisation, resulting in reduced nitrification and the risk of N loss (Abalos et al., 2019; Bengtson et al., 2012; Fisk et al., 2015). This was not the case in this study, probably because the high rates of added N could have increased nitrification. Indeed, there was a strong positive correlation between soil NH_4^+-N concentrations and N_{nit}, which corroborates that NH_4^+ availability is a major regulating factor for nitrification (Booth et al., 2005; Li et al., 2018). Even if an increase in C rhizodeposition induced by high N availability may have stimulated microbial N immobilisation, this effect was likely marginal compared to the effect of increased NH_4^+ supply on nitrification.

Contrary to previous findings where N cycling has been related to shifts in the PLFA-based microbial community composition (Cookson et al., 2007, 2005), this effect was not evident in this study. In an extensive investigation across various ecosystems, Graham et al. (2016) found that the soil microbial community was an inconsistent predictor for soil nitrification and N mineralisation. Instead, more of the variability was explained by edaphic factors, such as soil pH and NH_4^+ availability (E. B. Graham et al., 2014; Graham et al., 2016). This is of particular importance for systems with high N inputs, as Orwin et al. (2020) showed that N cycling indicators were decoupled from the soil microbial community in grassland systems exposed to N inputs of more than 200 kg N ha^{-1}. This is consistent with the findings from this study, where NH_4^+ availability was a better predictor for N_{min} and N_{nit} than the microbial community composition.

The lack of clear evidence for a linkage between $^{13}C_{we}$, C_{MB}, or $^{13}C_{MB}$ and N_{min} or N_{nit} may indicate a decoupling of soil C and N cycles when N inputs are excessive. It has been discussed that excessive N inputs from livestock urine and concomitant biomass removal through livestock grazing can lead to an decoupling of C and N cycles in intensive grassland systems (Rumpel et al., 2015; Soussana and Lemaire, 2014). This decoupling can result in reduced C and N retention (Lemaire et al., 2014), suggesting that the high N inputs and continuous biomass removal in this study could have increased the risk for N losses.

4.6 Conclusions

This study contributed new insights to C and N cycling in high N grassland systems by combining measurements of plant C balance with those of ^{15}N pool dilution and of ^{13}C isotopic composition of plant, soil, and microbial components after $^{13}CO_2$ pulse-labelling of planted microcosms. Differences in plant C balance and C rhizodeposition among the treatments of plant species and increasing N addition were associated with changes in the microbial community composition and microbial processing of rhizodeposited C. Differences in microbial uptake of rhizodeposited C between plant species varied with increasing N addition, highlighting that the fate of rhizodeposited C from different plant species can vary between low-N and high-N systems. While microbial community composition and the uptake of rhizodeposited C were closely associated with indicators of C cycling, the consequences of this for N cycling were unclear. This may suggest a decoupling of soil C and N cycles in the high N systems studied here, but further research is needed to test the applicability of the findings to field conditions.

Chapter 5

The effects of imbalances between microbial elemental requirements and available substrate stoichiometry on soil organic matter fractions and microbial community composition

5.1 Abstract

Grassland management practices can alter the elemental composition of both plant biomass and soil substrates. This can lead to an imbalance between available soil substrate stoichiometry and microbial biomass stoichiometry, likely constraining microbial elemental cycling. This study used the concept of ecological stoichiometry to link soil biogeochemistry with microbial cycling of carbon (C), nitrogen (N), and phosphorus (P) to explore the relationship between biogeochemical cycles and soil functional processes in an experimental grassland under different long-term (>25 years) plant biomass management practices. Plant and soil samples were collected from the treatments comprising never mown, frequently or infrequently mown with clippings retained, infrequently mown with clippings removed, and N addition (0 or 50 kg N ha^{-1}).

Continuous removal of plant biomass after mowing depleted soil available inorganic P, resulting in significantly higher soil C:P ratios compared to those in mown plots where plant biomass was retained. Across all experimental treatments, C:N and C:P ratios were both greater for the soil microbial biomass than for available soil substrates, suggesting that the soil microbial community was limited primarily by C. The microbial community adjusted its composition and metabolic enzyme production in response to stoichiometric imbalance of available substrates. Both the microbial community composition and microbial metabolic C limitation were related significantly to alterations in concentrations of soil organic matter (SOM) fractions and soil respiration rate. Despite experimental N addition and differences in soil N concentrations, the microbial community composition and stoichiometric elemental demand were not related to N mineralisation and nitrification rates. The finding that microbial C limitation was associated with the concentrations of SOM fractions and soil respiration may have important implications for the development of sustainable grassland management practices that promote SOM protection and increasing stocks.

5.2 Introduction

Globally, soil organic carbon (SOC) stocks in grasslands have decreased over the past 12,000 years – most dramatically in the recent 200 years – leading to a current estimated carbon (C) 'debt' of approximately 22.4 Pg C for the upper 2 m of soil (Sanderman et al., 2017). Because grasslands provide critical ecosystem services, such as nutrient retention and soil C storage, there is increasing attention given to improving their sustainability while maintaining high productivity (Conant et al., 2017; Fornara et al., 2016; Wilsey, 2018). One strategy identified as effective in enhancing these sustainable outcomes is increasing SOC stocks (Amelung et al., 2020; IPCC, 2019; Minasny et al., 2017). Management strategies that increase SOC stocks would benefit agricultural sustainability as well as climate change mitigation by removing atmospheric carbon dioxide (CO_2) (Dignac et al., 2017; Smith, 2008; Soussana et al., 2019). This is of particular interest for grasslands, because grasslands cover about one quarter of the terrestrial surface area, contain an estimated 20% of the world's SOC stock, and are easier to manage compared to other terrestrial ecosystems (Chabbi et al., 2017; FAO, 2018; Stockmann et al., 2013; Whitehead et al., 2018). To successfully implement such management strategies, evidence for how agricultural practices on grasslands influence SOC is needed urgently (Harden et al., 2018; Lavallee et al., 2019; Paustian et al., 2016).

Soil organic matter (SOM), which is typically comprised by 50 to 58% of SOC (Rayment and Lyons, 2011; Schlesinger, 1977), can accumulate when the formation of organic matter from inputs derived primarily from plants is protected and exceeds losses from decomposition (Jastrow et al., 2007). The components of SOM can be separated into a labile, fast-cycling pool and a stable, slow-cycling pool (Cotrufo et al., 2015; Lavallee et al., 2019; Lehmann and Kleber, 2015). Labile particulate organic matter (POM) consists mostly of relatively undecomposed plant components with a short turnover time (years to decades), but it can accumulate indefinitely (Cotrufo et al., 2019, 2013; Lavallee et al., 2019). In contrast, mineral-associated organic matter (MAOM), which is derived partly from microbial metabolites (Angst et al., 2021), can remain in the soil for centuries because the mineral-association reduces microbial accessibility and thereby protects MAOM against microbial turnover (Kirschbaum et al., 2020; Lavallee et al., 2019). However, MAOM formation is limited by C-saturation of mineral surfaces and by microbial production of low molecular weight compounds (Castellano et al., 2015; Cotrufo et al., 2019, 2013). While recent conceptual frameworks on SOM dynamics have considered that microbial biomass growth can be limited by the availability of nitrogen (N) (Averill and Waring, 2018; Castellano et al., 2015; Cotrufo et al., 2013), there has been much less emphasis on investigating other potentially limiting elements, such as phosphorus (P) on SOM dynamics and ecosystem functioning (Buchkowski et al., 2019; Peñuelas et al., 2012; Reed et al., 2015; Soong et al., 2020).

Ecological stoichiometry, which links the elemental (e.g. C:N:P) composition of microbial consumers with that of their substrates (Sterner and Elser, 2002), can aid in explaining SOM formation and its connection to the biogeochemical cycling of C, N, P, and other elements (Buchkowski et al., 2019;

Fatichi et al., 2019; Mooshammer et al., 2014b; Soong et al., 2020). In response to possibly imbalanced elemental ratios of available substrates, microbial communities can retain those elements which limit their growth and excrete those that are in excess (Mooshammer et al., 2014b; Zechmeister-Boltenstern et al., 2015). Because soil microorganisms excrete C as respired CO_2 and N through ammonium (NH_4^+) mineralisation, biogeochemical cycles and soil functional processes are influenced, directly and indirectly, by the stoichiometric balance that limits microbial productivity (Manzoni et al., 2008; Mooshammer et al., 2014b; Zechmeister-Boltenstern et al., 2015). Despite its importance, the role of microbial stoichiometry has often been overlooked in conceptual frameworks relating SOM dynamics to ecosystem functioning (Buchkowski et al., 2019; Soong et al., 2020).

In response to stoichiometric imbalances in available substrates, soil microbial communities could change their metabolic extracellular enzyme production (Mooshammer et al., 2014b). Sinsabaugh et al. (2009, 2008) used eco-enzymatic stoichiometry, that is the activity ratios of C-acquiring enzymes relative to those for N- or P-acquiring enzymes, to show that the mean ratio of microbial C:N:P acquisition across ecosystems globally is close to 1:1:1. This was hypothesised to represent stoichiometric equilibrium between microbial demand for certain elements and their availability from the soil. Based on this, Moorhead et al. (2016) suggested using vector analysis on proportional C:N- versus C:P-acquiring enzyme activities to quantify relative metabolic C, N, and P limitations. This method has been used widely to quantify microbial investment in C relative to investment in N and P acquisition (e.g. Chen et al., 2019; Cui et al., 2020; Fanin et al., 2016; Forstner et al., 2019; Keane et al., 2020; Kuske et al., 2019; Liu et al., 2020; Pei et al., 2017).

The long time needed for stoichiometric effects related to grassland management practices to be realised may preclude short- or medium-term experiments (Loughin et al., 2007). Long-term ecosystem experiments can provide insight into the whole trajectory of how an ecosystem and its components respond to a certain treatment (Knapp et al., 2012). Here, an established long-term grassland experiment was studied to investigate the relationships between microbial community composition and function, microbial elemental demand, and substrate stoichiometry influenced by continuous grassland management. The objective was to assess how microbial communities limited by available C, N, or P affect SOM concentrations and soil functional processes (basal respiration and gross N transformation rates). The conceptual framework for this study is based on the model proposed by Zechmeister-Boltenstern et al. (2015) where substrate stoichiometry influences the microbial community composition and their function (Figure 5.1). The hypotheses were that (1) continuous removal of biomass after mowing and no N addition would imbalance the C:N:P stoichiometry of substrates available to microbial communities, (2) in response to the stoichiometric imbalance between available substrates and microbial elemental requirements, the microbial community would adjust its composition and the production of extracellular enzymes suited to the acquisition of the limiting element, and (3) microbial elemental limitation would covary with concentrations of SOM fractions and rates of soil respiration and N transformations.

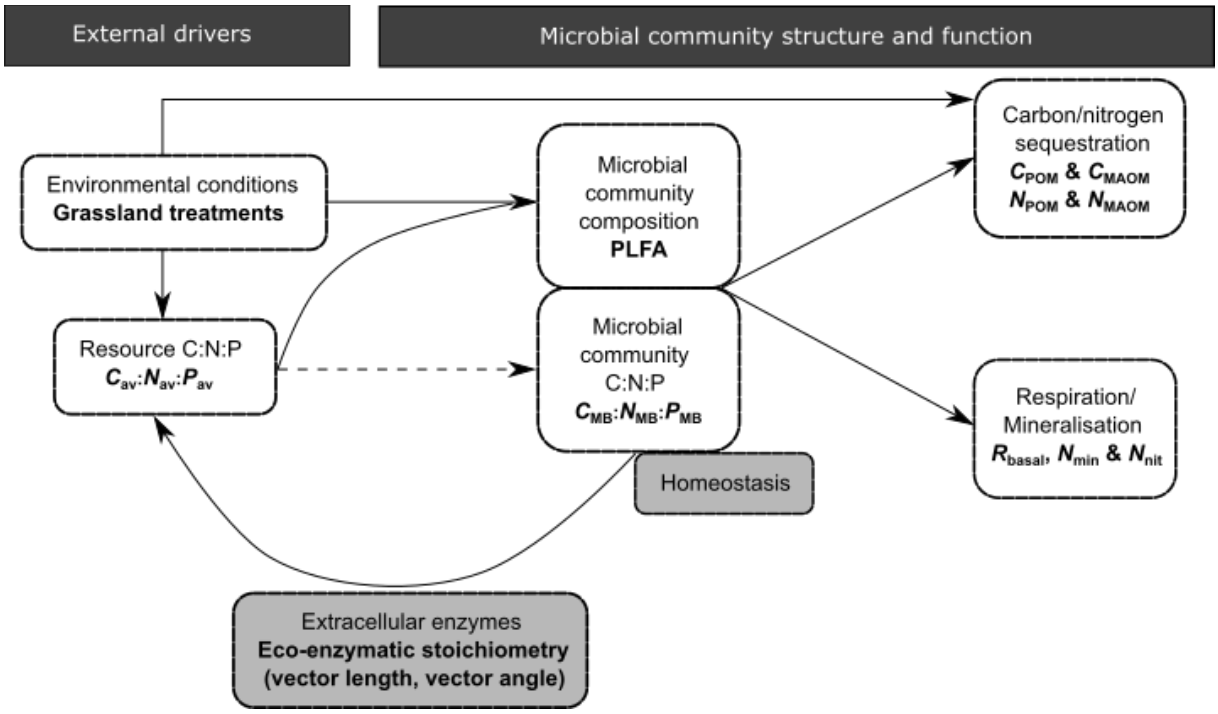

Figure 5.1. Conceptual diagram of hypothesised effects of substrate stoichiometry on microbial community composition and function (adapted from Zechmeister-Boltenstern et al., 2015). The variables relating to ecosystems more generally as indicated by the authors are shown in non-bold text, while corresponding variables used in this study are shown in bold text. C_{av}:N_{av}:P_{av} = carbon (C):nitrogen (N):phosphorus (P) ratio of available soil substrates; PLFA = phospholipid fatty acids, a measure of microbial community composition; C_{MB}:N_{MB}:P_{MB} = C:N:P ratio of microbial biomass; C_{POM} and N_{POM} = C and N concentrations of particulate organic matter fraction; C_{MAOM} and N_{MAOM} = C and N concentrations of mineral-associated organic matter fraction; R_{basal} = basal soil respiration rate; N_{min} = gross N mineralisation rate; N_{nit} = gross nitrification rate. Solid and dashed lines indicate direct and indirect influences, respectively. Grey boxes represent underlying principles and adjustments by the microbial community to elemental imbalances.

5.3 Materials and Methods

5.3.1 Site description

The long-term ecology trial (LTET) was established in September 1994 at Lincoln University, New Zealand (latitude -43.648' S, latitude 172.469' E; 14 m above sea level), to study grassland management effects on insect and plant ecology as well as on soil properties (Farrell et al., 2014; Simpson et al., 2012). The trial is on a Wakanui silt loam (Mottled Immature Pallic (New Zealand Soil Classification, NZSC, Hewitt, 2010); Udic Ustochrept (USDA, Soil Survey Staff, 2014)). The trial comprises 32 plots (5 m × 5 m) arranged in a randomised block design, initially cultivated and sown with red clover (*Trifolium repens* L. cv. 'Pawera'), white clover (*Trifolium repens* L. cv. 'Tahora'), perennial ryegrass (*Lolium perenne* L.), and cocksfoot (*Dactylis glomerate* L. cv. 'Kahu'). The plots were exposed to 8

different treatment combinations (4 replicates) comprising a biomass removal treatment by mowing (4 levels) and a N fertiliser application treatment (2 levels) (Table 5.1). The infrequent mowing treatment was carried out when the sward reached a height of about 300 mm, while the frequent mowing treatment was carried out at a sward height of 200 mm. Nitrogen fertiliser was applied annually as urea in September (early spring) at a rate of either 0 or 50 kg N ha^{-1}.

The treatments have remained unchanged since trial establishment, which resulted in shifts in plant community composition among the treatments (Adair et al., 2013). For example, clover species disappeared in the never mown treatments as they were outcompeted for light by taller grass species. Simultaneously, other plant species invaded the mown treatments, such as common daisy (*Bellis perennis* L.) and ribwort plantain (*Plantago lanceolata* L.). In 2011, the dominant species on the mown treatment plots were white clover, perennial ryegrass, cocksfoot, yarrow (*Achillea millefolium* L.) and chickweed (*Stellaria media* (L.) Vill.), whereas cocksfoot was the sole dominant species on the never mown treatments (Dignam et al., 2019). Values for biomass production are shown in Table A.3.1 (Appendix A.3).

Table 5.1. Experimental design of the long-term ecology trial with biomass and N addition treatment combinations and respective treatment symbols/abbreviations. The frequently and infrequently mown plots were mown when the swards reached a height of 200 and 300 mm, respectively.

Treatment identifier	Biomass treatment	N addition treatment (kg N ha^{-1} y^{-1})
$M_iC_0N_0$	Infrequently mown (M_i) & clippings removed (C_0)	0 (N_0)
$M_iC_0N_1$	Infrequently mown (M_i) & clippings removed (C_0)	50 (N_1)
$M_iC_1N_0$	Infrequently mown (M_i) & clippings retained (C_1)	0 (N_0)
$M_iC_1N_1$	Infrequently mown (M_i) & clippings retained (C_1)	50 (N_1)
$M_fC_1N_0$	Frequently mown (M_f) & clippings retained (C_1)	0 (N_0)
$M_fC_1N_1$	Frequently mown (M_f) & clippings retained (C_1)	50 (N_1)
M_0N_0	Never mown (M_0)	0 (N_0)
M_0N_1	Never mown (M_0)	50 (N_1)

5.3.2 Plant sampling and analyses

About 25 years after trial establishment, plant and soil samples were collected on four consecutive days from 30 October to 2 November 2019. During this time, the weather was relatively stable with similar daytime temperatures ranging from 14 to 21 °C and no significant precipitation.

Shoot biomass was sampled by removing aboveground biomass within a 0.196 m^2 circular frame placed randomly on each treatment plot. Root samples were taken by inserting a soil corer (47.6 mm diameter) to a depth of 100 mm on the spots where shoot biomass was sampled earlier to minimize disturbance of the trial. Two cores were taken on each treatment plot, bulked, and roots washed carefully. The dry mass of the shoots and roots were weighed after drying at 65 °C for 120 h. A dried and ground subsample was analysed for shoot and root C and N concentrations (C_{shoot}, N_{shoot}, C_{root}, N_{root}) on an elemental analyser (Elementar Vario-Max CN Elemental Analyser, Elementar GmbH, Hanau, Germany) and shoot and root P concentrations (P_{shoot}, P_{root}) by ICP-OES (Varian 720-ES, Varian Inc., Palo Alto, California, USA) after microwave digestion using nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) (Juranović Cindrić et al., 2015).

5.3.3 Soil sampling and chemical analyses

On each plot, 15 to 20 soil cores (23 mm diameter) were collected from random locations to a depth of 100 mm and bulked. The soil was sieved in the field (≤ 4 mm) to remove roots and other large particles and stored at 4 °C until further processing.

Gravimetric soil water content was determined by mass loss after drying at 105 °C for 24 h. Soil pH was measured in 0.01 M $CaCl_2$ (1:2.5 w:v) (Hendershot et al., 2008; Miller and Kissel, 2010). Concentrations of NH_4^+-N and NO_3^--N were measured by flow injection analysis (FOSS FIAstar 5000, Foss Tecator AB, Hoganas, Sweden) after extraction from a fresh soil sample with 2 M KCl (1:10 w:v) (Rayment and Lyons, 2011). Concentrations of total organic C (C_t) and total N (N_t) were analysed by dry combustion on an elemental analyser (Elementar GmbH, Hanau, Germany), whereas total P concentration (P_t) was measured by ICP-OES (Varian 720-ES, Varian Inc., Palo Alto, California, USA) after microwave digestion with aqua regia (Rayment and Lyons, 2011). Dissolved organic C and dissolved N were extracted with 0.5 M K_2SO_4 (1:4, w:v) for 30 min and concentrations measured (Shimadzu TOC Analyser model 5000A with ASI-5000A, Shimadzu Oceania Pty Ltd., Sydney, Australia). The dissolved organic C and dissolved N concentrations were considered as the soil C and N fractions available for microbial uptake and designated as C_{av} and N_{av}, respectively. Microbially available P was extracted from a fresh soil sample with 0.5 M $NaHCO_3$ (1:100 w:v) at pH 8.5 for 16 h (Colwell, 1963; Rayment and Lyons, 2011). The P concentration (P_{av}) in the extracts was measured following the colorimetric method of Dick and Tabatabai (1977) modified by He and Honeycutt (2005).

5.3.4 Soil organic matter fractionation

Soil organic matter was fractionated by particle size following the method of Castellano et al. (2012) with modifications of Cotrufo et al. (2019) and Poeplau et al. (2018). Briefly, 20 g of air-dried soil was dispersed in dilute sodium hexametaphosphate (0.5 % $(NaPO_3)_6$) by reciprocal shaking for 16 h at room temperature. The mixture was then poured on to a sieve with 53 µm mesh size and rinsed with deionized water until the water draining from the sieve appeared clear against a white background. Both fractions were dried at 60 °C and the C and N concentrations in each fraction measured on an elemental analyser (Elementar GmbH, Hanau, Germany). Carbon and N contained in particles >53 µm were operationally defined as components of particulate organic matter (POM) and their concentrations designated as C_{POM} and N_{POM}, respectively. Carbon and N contained in particles <53 µm were operationally defined as components of mineral-associated organic matter (MAOM) and their concentrations designated at C_{MAOM} and N_{MAOM}, respectively.

5.3.5 Gross N transformation rates

Gross N transformation rates were determined using ^{15}N labelled substrates in a pool-dilution approach with paired treatments (Kirkham and Bartholomew, 1954; Murphy et al., 2003) as described by Bengtson et al. (2003) and Braun et al. (2018). Briefly, 7.7 g of fresh soil was mixed with 300 µL of a tracer solution containing 1.75 mmol L^{-1} $(NH_4)_2SO_4$ and 0.57 mmol L^{-1} KNO_3, but ^{15}N-labelled either on NH_4^+ (98% $^{15}N_2$; Cambridge Isotope Laboratories, MA, USA) or on NO_3^- (99% ^{15}N; Cambridge Isotope Laboratories, MA, USA), and incubated at 22 °C in the dark. To minimise stimulation of microbial N consumption induced by the added substrate, a tracer concentration was chosen that limited the increase in NH_4^+ and NO_3^- concentrations by less than 25%, while still leading to a sufficient enrichment with ^{15}N (Davidson et al., 1991).

To determine the initial proportion of ^{15}N in the samples, a subset of the incubated samples was extracted with 2 M KCl (1:5 w:v) (Rayment and Lyons, 2011) after allowing the tracer to equilibrate for 2 h (Braun et al., 2018; Murphy et al., 2003). The other subset was extracted 24 h after tracer addition. The extracts were stored at -20 °C until further processing. Extracted NH_4^+ and NO_3^- concentrations were measured by flow injection analysis (FOSS FIAstar 5000, Foss Tecator AB, Hoganas, Sweden). For analysis of $^{15}N/^{14}N$ isotopic composition in NH_4^+ and NO_3^-, the soil extracts were prepared using a sequential microdiffusion approach (Keeney and Nelson, 1982; Sørensen and Jensen, 1991). Ammonium and NO_3^- were trapped sequentially on separate acidified filter disks after conversion to NH_3 with MgO and Devarda's Alloy, respectively. Subsequently, the filter disks were analysed for $^{15}N/^{14}N$ isotopic composition on an elemental analyser (Sercon GSL, Crewe, UK) interfaced with a continuous flow-isotope ratio mass spectrometer (IRMS) (Sercon 20-22, Sercon, Crewe, UK). Gross N transformation rates were calculated following Wessel & Tietema (1992).

5.3.6 Soil microbial biomass-C, -N-, and -P

Soil microbial biomass-C (C_{MB}), -N (N_{MB}), and -P (P_{MB}) were determined using the chloroform fumigation-extraction method (Brookes et al., 1985, 1982; Vance et al., 1987) with modifications following Scott-Denton et al. (2006). Ethanol-free chloroform ($CHCl_3$) was added directly to a fresh soil sample (2:1 w:v) and incubated for 24 h at room temperature. After incubation, the samples were evacuated several times before extraction with 0.5 M K_2SO_4 (1:4, w:v) for C_{MB} and N_{MB} or 0.5 M $NaHCO_3$ (pH 8.5; 1:15 w:v) for P_{MB} by shaking for 30 min on an end-over-end shaker. Another set of samples was extracted equally but without prior chloroform fumigation. To correct for adsorption of released microbial P to soil particles, a third set of samples was extracted as described above but with addition of a P spike equivalent to 25 mg P kg^{-1}. The K_2SO_4 extracts were analysed for total organic C and total dissolved N (Shimadzu TOC Analyser model 5000A with ASI-5000A, Shimadzu Oceania Pty Ltd., Sydney, Australia), while the $NaHCO_3$ extracts were analysed as above. The difference in C, N, and P concentrations between the fumigated and non-fumigated samples was used to quantify soil C_{MB}, N_{MB}, and P_{MB}, respectively, after applying extraction efficiency correction factors k_{EC} = 0.45 (Vance et al., 1987), k_{EN} = 0.54 (Brookes et al., 1985), and k_{EP} = 0.40 (Brookes et al., 1982).

5.3.7 Soil microbial community composition

The microbial community composition was characterised by phospholipid fatty acid (PLFA) biomarker analysis (Frostegård et al., 2011, 1993) after PLFA extraction, fractionation, and methylation (Bligh and Dyer, 1959; Frostegård et al., 1991; White et al., 1979) using the method of Quideau et al. (2016). Lipids were extracted by eluting 4.0 g freeze-dried soil in chloroform, methanol, and citrate buffer (1:2:0.8 v:v:v) and phospholipids were separated by a silica-bonded solid phase extraction column. Mild alkaline methanolysis was used to methylate phospholipids to form fatty acid methyl esters (FAME), which were analysed by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu GCMS-QP2010 (Shimadzu Oceania Pty Ltd, Sydney, Australia) fitted with a Restek Rtx-5ms fused silica capillary column (30.0 m × 0.25 mm i.d. × 0.25 µm; Bellefonte, PA, USA). For each analysis, 1 µL of sample solution was injected into the GC injection port operating at a pressure of 240 kPa for 2 min at 250 °C with a split ratio of 20:1. Oven temperature was kept constant at 150 °C for 3 min, then increased to 200 °C at 1.5 °C min^{-1} and to 240 °C at 4 °C min^{-1}. After final heating to 300 °C at 30 °C min^{-1}, the temperature was held for 7 min. Helium was used as a carrier gas with a constant linear velocity of 0.38 m sec^{-1} in split mode (1 mL min^{-1}). For mass spectrometry (MS), the electron energy was 70 eV and the scanning range of mass-to-charge ratio (m/z) was set from 35 to 500. The temperature of the capillary interface was 310 °C and the MS source temperature was 260 °C.

The concentration of detected FAMEs was calculated by including two internal standards (C 13:0 and C 19:0) in the analysis. A mix of bacterial fatty acid methyl esters (Supelco 47080-U, Sigma-Aldrich) was used to identify sample peaks by comparing retention times. The following nomenclature was used to designate identified PLFA to microbial groups: PLFAs 14:0, 15:0, 16:0, and 18:0 as general bacterial biomarkers, PLFAs i15:0, a15:0, i16:0, i17:0, and a17:0 for gram-positive bacteria, PLFAs 16:1ω7, cy17:0, and cy19:0 for gram-negative bacteria (Waldrop and Firestone, 2004; Zelles, 1999, 1997), PLFAs 18:2ω6,9, 18:3ω6,9,12, and 18:1ω9 as general fungal biomarkers (Frostegård and Bååth, 1996; Stahl and Klug, 1996; Vestal and White, 1989), and the PLFAs 10Me16:0 and 10Me18:0 for actinomycetes (Vestal and White, 1989). The PLFA 16:1ω5 was used as an indicator for arbuscular mycorrhizal fungi (AMF) (Olsson, 1999). However, since it was also found in gram-negative bacteria (Ruess and Chamberlain, 2010), this PLFA was interpreted carefully.

5.3.8 Basal soil respiration rate

Basal soil respiration rate (R_{basal}) was measured by incubating a fresh soil sample equivalent to 3 g dry mass in a sealed 12 mL vial for 1 h at 25 °C in the dark. The gas in the vial headspace air was sampled at the start of the incubation and again after 1 h using a syringe and the CO_2 partial pressure was measured by injecting the sample into a stream of CO_2-free air flowing into an infra-red gas analyser (Model LI-7000, LICOR Inc., Lincoln, NE, USA). The respiration rate was derived from the difference in CO_2 partial pressure in the headspace air between the start and the end of the incubation period.

5.3.9 Extracellular enzyme activities

The potential activities of four common enzymes involved in soil C, N, and P cycling processes were determined (Sinsabaugh et al., 2009). The activities of β-1,4-glucosidase (BG, EC 4.2.1.21), β-1,4-N-acetylglucosaminidase (NAG, EC 3.2.1.14), leucine aminopeptidase (LAP, EC 3.4.11.1), and acid phosphatase (AP, EC 3.1.3.1) were quantified colorimetrically with *para*-nitrophenyl linked substrates (Eivazi and Tabatabai, 1988, 1977; Parham and Deng, 2000; Tabatabai and Bremner, 1969) as described by Deng and Popova (2011) and Acosta-Martínez and Tabatabai (2011). For each sample, 1.0 g fresh soil was mixed with 0.2 mL toluene, 4 mL of modified universal buffer or 100 mM acetate buffer adjusted to the respective pH, and 1 mL of respective substrate solution (Table A.3.2, Appendix A.3) and incubated at 37 °C for 1 h. After incubation, the enzymatic reaction was terminated by adding 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH (for acid phosphatase) or 0.1 M tris(hydroxymethyl)aminomethane (THAM) buffer (pH 12) (for all other enzymes) and filtered. The colour intensity of the filtrate was measured with a spectrophotometer at wavelength 405 nm.

5.3.10 Stoichiometric imbalance and metabolic elemental limitation

The stoichiometric imbalance between available substrates and the microbial elemental demand was derived by calculating the log-transformed ratio of substrate stoichiometry (C:N_{av} or C:P_{av}) and microbial biomass stoichiometry (C:N_{MB} or C:P_{MB}) (Mooshammer et al., 2014a).

The metabolic response of the microbial community to imbalanced substrate stoichiometry was estimated from the relative microbial investments in enzymes for energy (C) vs. N and P acquisition and for P vs. N acquisition. A quantitative vector analysis on proportional extracellular enzyme activities was used (Moorhead et al., 2016). The proportional activities of C- vs. P- and C- vs. N-acquiring enzymes were calculated as BG/(BG + AP) and BG/(BG + NAG + LAP), respectively. When plotted, the position of C vs. N against C vs. P mineralising enzyme activities indicates the relative microbial investment in C, N, and P acquisition (DeForest and Moorhead, 2020). Vector length between this position and the plot origin indicates the relative microbial investment in C vs. N and P acquisition (equation 5.1):

$$Vector\ length = \sqrt{\left[\frac{BG}{BG + AP}\right]^2 + \left[\frac{BG}{BG + NAG + LAP}\right]^2} \tag{5.1}$$

Metabolic C limitation increases with increasing vector length. The angle between the vector and the *x*-axis (i.e., C vs. P enzyme activity) indicates relative N- vs. P-acquiring enzyme activities (equation 5.2):

$$Vector\ angle = \arctan\left(\left[\frac{BG}{BG + AP}\right], \left[\frac{BG}{BG + NAG + LAP}\right]\right) \tag{5.2}$$

A vector angle > 45° is indicative for P limitation, while vector angles < 45° imply N limitation. Increasing vector angles represent increasing P limitation and decreasing N limitation, respectively (Moorhead et al., 2016).

Ratios of BG:(NAG+LAP) and (NAG+LAP):AP were plotted against each other to indicate potential elemental co-limitation based on eco-enzymatic stoichiometry (Hill et al., 2012).

5.3.11 Statistical analyses

All elemental and microbial ratios were log-transformed prior to statistical analysis to avoid skewness of the data (Isles, 2020). Statistical analyses were performed in R v. 4.0.3 (R Core Team, 2020). For all statistical analyses, *P*-values less than 0.05 were considered significant.

The effects of biomass (4 levels) and N addition (2 levels) treatments and their interaction on plant and soil properties and processes (C_{shoot}, N_{shoot}, P_{shoot}, C_{root}, N_{root}, P_{root}, pH, C_t, N_t, P_t, C_{av}, N_{av}, P_{av}, C_{MB}, N_{MB}, P_{MB}, C_{POM}, C_{MAOM}, R_{basal}, N_{min}, N_{nit}) were analysed using two-way factorial analysis of variance (ANOVA) for multiple linear regression models. Significant differences ($P < 0.05$) between group effect estimates were investigated with Tukey HSD post-hoc tests for multiple comparisons of all biomass and N addition treatments. To correct for multiplicity (i.e., to control type I error) when making many simultaneous inferences, the '*multcomp*' package was used (Hothorn et al., 2008). Assumptions of normality of the residuals and homoskedasticity were confirmed by visual assessment of residual plots and plots of predicted vs. observed values. In cases of heteroskedasticity, linear models were refitted using the sandwich estimator of the covariance matrix (Zeileis, 2006, 2004). Log-transformations of data were used as required to meet the assumptions.

Linear regression was used to test for relationships between measured variables, stoichiometric ratios, and microbial community indices (i.e., fungal:bacterial ratio, gram-positive:gram-negative bacterial ratio). Relationships among available elemental ratios (C:N_{av} and C:P_{av}) and vector length or vector angle were investigated by using standardised major axis (SMA) analysis (Warton et al., 2006) using the '*lmodel2*' package (Legendre, 2018). Standardised major axis regression estimates the best fit bivariate line between two variables and thereby accounts for the uncertainty in both variables. Residual checks were used throughout.

Relative abundances of PLFA biomarkers (mol%) were used to analyse the microbial community composition. Permutational multivariate analysis of variance (PERMANOVA) was used with 999 within-block permutations to assess the effects of biomass and N addition treatments on microbial community composition. Subsequently, the assumption of multivariate homogeneity of group dispersion was confirmed using the method proposed by Anderson (2006). Non-metric multidimensional scaling (NMDS) ordination with Bray-Curtis distances was used to visualise the microbial community data (McCune and Grace, 2002). All data were standardised using the Wisconsin double standardisation procedure. Vector fitting was used to test for linear relationships between the NMDS ordinated microbial community and environmental variables. All microbial community data analyses were performed using the '*vegan*' package (Oksanen et al., 2019).

5.4 Results

5.4.1 Plant and soil properties

Among the plant elemental concentrations, only N_{shoot}, P_{shoot}, and P_{root} were affected significantly by the different biomass removal treatments (Table 5.2). Compared to that for M_0, mean N_{shoot} was increased by 5.88 (P = 0.001) and 5.38 mg N g^{-1} (P = 0.003) for M_fC_1 and M_iC_1, respectively, while that for M_iC_0 was not significantly different (P = 0.466). For P_{shoot}, mean values for M_fC_1 and M_iC_1 were increased by 1.58 (P < 0.001) and 1.34 mg P g^{-1} (P < 0.001), respectively, while those for M_iC_0 were not significantly different compared to those for M_0 (P = 0.057). Similarly, mean P_{root} values were 0.881 (P = 0.001) and 0.736 mg P g^{-1} (P = 0.005) higher for M_fC_1 and M_iC_1, respectively, than those for M_0, while those for M_iC_0 were similar to those for M_0 (P = 0.986). These significantly higher values of N_{shoot}, P_{shoot}, and P_{root} indicate enhanced aboveground and belowground litter quality (lower C:N_{shoot}, C:P_{shoot}, C:P_{root}). The addition of N fertiliser led to an increase in mean N_{shoot} by 2.52 mg N g^{-1} (P = 0.040) compared to those without N addition.

Table 5.2. Mean ± standard deviation of plant properties for each experimental biomass and N addition treatment (see Table 5.1 for treatment descriptions). Significant *F*- and *P*-values (ANOVA) for treatment effects on plant properties are included. Interactive treatment effects were never significant and therefore omitted. NS = not significant.

Response variables (unit)	Treatment								*Biomass treatment*	*N addition*
	$M_fC_1N_0$	$M_fC_1N_1$	$M_iC_1N_0$	$M_iC_1N_1$	$M_iC_0N_0$	$M_iC_0N_1$	M_0N_0	M_0N_1	*F* (*P*)	*F* (*P*)
C_{shoot} (mg C g^{-1})	402.8 ± 4.8	402.8 ± 6.6	399.1 ± 5.7	400.9 ± 8.4	407.7 ± 3.9	404.2 ± 11.6	402.7 ± 6.1	413.1 ± 6.7	NS	NS
N_{shoot} (mg N g^{-1})	25.4 ± 3.0	25.9 ± 2.4	21.8 ± 3.6	28.6 ± 3.7	20.3 ± 1.8	21.9 ± 3.9	19.3 ± 3.8	20.2 ± 2.2	6.31 (0.002)	4.66 (0.040)
P_{shoot} (mg P g^{-1})	3.74 ± 0.18	3.88 ± 0.14	3.83 ± 0.72	3.32 ± 0.41	2.86 ± 0.56	2.62 ± 0.45	2.03 ± 0.32	2.43 ± 0.76	18.64 (< 0.001)	NS
C_{root} (mg C g^{-1})	400.9 ± 19.6	400.7 ± 4.1	400.5 ± 7.1	392.0 ± 7.7	380.4 ± 17.1	395.5 ± 10.5	382.7 ± 20.1	405.3 ± 5.4	NS	NS
N_{root} (mg N g^{-1})	11.4 ± 2.0	13.8 ± 3.4	10.6 ±1.7	13.4 ± 1.4	11.4 ± 0.9	11.7 ± 1.8	12.2 ± 2.6	11.0 ± 0.7	NS	NS
P_{root} (mg P g^{-1})	1.86 ± 0.58	2.33 ± 0.22	2.34 ± 0.57	1.65 ± 0.28	1.55 ± 0.28	1.50 ± 0.34	1.12 ± 0.78	1.31 ± 0.42	5.53 (0.004)	NS

Soil pH was affected significantly by long-term biomass and N addition treatments (Table 5.3). The mean soil pH was lower by 0.148, 0.161, and 0.249 units for M_fC_1, M_iC_1, and M_iC_0, respectively, compared to that for M_0. Addition of N fertiliser slightly decreased mean soil pH by 0.076 units ($P = 0.004$) compared to that for N_0. Retention of clippings clearly increased mean C_t and N_t, which were 4.51 ($P = 0.001$) and 5.78 g C kg^{-1} ($P < 0.001$) and 0.539 ($P < 0.001$) and 0.556 g N kg^{-1} ($P < 0.001$) higher for M_fC_1 and M_iC_1, respectively, compared to the values for M_0. In contrast, there was no significant difference in mean P_t between the values for M_0, M_fC_1, and M_iC_l, but values for M_iC_0 were decreased by 0.0138 g P kg^{-1} ($P = 0.016$) than those for M_0. While there was no significant difference in mean C_{av} between the biomass ($P = 0.937$) or N addition treatments ($P = 0.141$), mean N_{av} was reduced significantly in M_0 by 9.72 ($P = 0.011$) and 13.94 mg N kg^{-1} ($P < 0.001$) compared to those for M_fC_1 and M_iC_1, respectively. For M_iC_0, mean P_{av} was strongly depleted by 1.40 mg P kg^{-1} ($P < 0.001$) and mean N_{av} slightly (not significantly) decreased by 5.34 mg N kg^{-1} ($P = 0.145$) compared to those for M_0. Mean concentrations of SOM fractions in the grassland soils studied here varied significantly between the biomass treatments. As expected, mean C_{POM} and N_{POM}, were higher for M_fC_1 and M_iC_1 by 1.09 ($P = 0.068$) and 2.11 g C kg^{-1} ($P < 0.001$) and 0.116 ($P = 0.019$) and 0.188 g N kg^{-1} ($P < 0.001$), respectively, compared to those for M_0. Similarly, mean C_{MAOM} and N_{MAOM} were greater by 1.82 ($P < 0.001$) and 1.44 g C kg^{-1} ($P = 0.006$) and 0.233 ($P < 0.001$) and 0.210 g N kg^{-1} ($P < 0.001$) for M_fC_1 and M_iC_1, respectively, than those for M_0.

Mean C_{MB} was significantly lower in M_0 by 68.9 ($P = 0.003$), 110.6 ($P < 0.001$), and 120.9 mg C kg^{-1} ($P < 0.001$) than those in M_iC_0, M_fC_1, and M_iC_1, respectively (Table 5.4). Also, mean N_{MB} was lower for M_0 compared to those for M_iC_0, M_fC_1, and M_iC_1 by 22.9 ($P = 0.003$), 29.0 ($P < 0.001$), and 27.2 mg N kg^{-1} ($P < 0.001$), respectively. Contrary to this, mean P_{MB} was not significantly different between the treatments. There was no significant effect of the biomass and N addition treatments on the soil functional processes, R_{basal} and N_{min}. However, mean N_{nit} was significantly greater for N_1 by 52% of that for N_0 but was not significantly different between the biomass treatments.

Table 5.3. Mean ± standard deviation of soil properties for each experimental biomass and N addition treatment (see Table 5.1 for treatment descriptions). Significant *F*- and *P*-values (ANOVA) for treatment effects on soil properties are included. Interactive treatment effects were never significant and therefore omitted. NS = not significant.

Response variables (unit)	Treatment								*Biomass treatment*	*N addition*
	$M_fC_1N_0$	$M_fC_1N_1$	$M_iC_1N_0$	$M_iC_1N_1$	$M_iC_0N_0$	$M_iC_0N_1$	M_0N_0	M_0N_1	*F* (*P*)	*F* (*P*)
pH	4.73 ± 0.05	4.64 ± 0.05	4.73 ± 0.11	4.62 ± 0.07	4.62 ± 0.03	4.55 ± 0.04	4.86 ± 0.12	4.81 ± 0.09	17.6 (< 0.001)	9.64 (0.005)
C_t (g C $kg^{-1)}$	32.2 ± 1.5	35.5 ± 2.62	33.9 ± 3.97	35.6 ± 3.5	29.6 ± 2.3	30.2 ± 2.5	29.2 ± 1.7	29.5 ± 1.4	10.9 (< 0.001)	NS
N_t (g N $kg^{-1)}$	3.13 ± 0.10	3.35 ± 0.12	3.13 ± 0.09	3.33 ± 0.25	2.78 ± 0.19	2.75 ± 0.24	2.68 ± 0.14	2.73 ± 0.29	19.8 (< 0.001)	NS
P_t (g P $kg^{-1)}$	0.585 ± 0.046	0.621 ± 0.057	0.694 ± 0.142	0.625 ± 0.028	0.589 ± 0.060	0.527 ± 0.031	0.646 ± 0.065	0.639 ± 0.074	3.65 (0.025)	NS
C_{av} (mg C kg^{-1})	106.7 ± 9.4	123.2 ± 5.2	105.4 ± 6.5	127.1 ± 8.9	106.8 ± 9.0	120.0 ± 8.9	112.8 ± 32.9	107.7 ± 16.1	NS	NS
N_{av} (mg N kg^{-1})	45.3 ± 5.8	53.1 ± 8.7	52.3 ± 8.1	53.1 ± 7.4	44.9 ± 3.6	44.8 ± 7.5	39.7 ± 8.1	39.4 ± 8.7	5.59 (0.004)	NS
P_{av} (mg P kg^{-1})	72.1 ± 19.4	76.0 ± 25.1	70.2 ± 28.7	75.9 ± 13.9	20.1 ± 11.2	19.8 ± 2.0	84.4 ± 25.4	72.0 ± 9.5	34.1 (< 0.001)	NS
C_{POM} (g C kg^{-1})	5.97 ± 0.90	7.82 ± 1.10	7.37 ± 1.25	8.49 ± 0.60	5.96 ± 1.21	5.74 ± 1.55	6.14 ± 0.68	5.48 ± 0.89	6.19 (0.002)	NS
N_{POM} (g N kg^{-1})	0.513 ± 0.097	0.635 ± 0.093	0.610 ± 0.104	0.680 ± 0.083	0.485 ± 0.094	0.445 ± 0.111	0.480 ± 0.058	0.435 ± 0.062	7.54 (< 0.001)	NS
C_{MAOM} (g C kg^{-1})	21.8 ± 1.0	22.8 ± 0.8	21.5 ± 0.9	22.4 ± 0.3	20.3 ± 1.2	20.6 ± 1.3	20.4 ± 1.3	20.6 ± 1.0	7.96 (< 0.001)	NS
N_{MAOM} (g N kg^{-1})	2.22 ± 0.09	2.30 ± 0.06	2.21 ± 0.04	2.26 ± 0.04	2.04 ± 0.10	2.09 ± 0.11	2.00 ± 0.12	2.05 ± 0.09	16.4 (< 0.001)	NS

Table 5.4. Mean ± standard deviation of microbial variables for each experimental biomass and N addition treatment (see Table 5.1 for treatment descriptions). Significant *F*- and *P*-values (ANOVA) for treatment effects on microbial variables are included. Interactive treatment effects were never significant and therefore omitted. NS = not significant.

Response variables (unit)	Treatment								*Biomass treatment*	*N addition*
	$M_fC_1N_0$	$M_fC_1N_1$	$M_iC_1N_0$	$M_iC_1N_1$	$M_iC_0N_0$	$M_iC_0N_1$	M_0N_0	M_0N_1	*F* (*P*)	*F* (*P*)
C_{MB} (mg C kg^{-1})	375 ± 37	374 ± 51	406 ± 46	370 ± 21	337 ± 51	329 ± 49	263 ± 41	265 ± 53	16.1 (< 0.001)	NS
N_{MB} (mg N kg^{-1})	67.6 ± 18.6	66.4 ± 18.6	61.5 ± 12.6	65.6 ± 18.1	61.2 ± 11.4	60.6 ± 10.2	38.4 ± 12.5	37.6 ± 14.8	7.30 (< 0.001)	NS
P_{MB} (mg P kg^{-1})	25.1 ± 7.1	21.6 ± 7.0	20.3 ± 9.9	26.1 ± 9.7	20.3 ± 5.2	19.7 ± 6.6	19.4 ± 3.6	14.9 ± 8.1	NS	NS
R_{basal} (µg CO_2-C g^{-1} h^{-1})	1.66 ± 0.23	1.70 ± 0.37	1.99 ± 0.16	1.54 ± 0.27	1.92 ± 0.34	1.62 ± 0.38	1.39 ± 0.30	1.46 ± 0.21	NS	NS
N_{min} (µg N g^{-1} d^{-1})	3.93 ± 0.34	5.25 ± 0.83	4.93 ± 1.46	3.30 ± 1.15	3.96 ± 2.03	3.00 ± 2.54	3.73 ± 0.50	5.05 ± 1.44	NS	NS
N_{nit} (µg N g^{-1} d^{-1})*	1.65 ± 1.00	2.87 ± 0.21	2.18 ± 1.40	3.53 ± 2.70	0.85 ± 0.83	1.38 ± 0.33	1.79 ± 0.17	1.08 ± 0.13	NS	4.92 (0.035)

* log-transformed

5.4.2 Effects of grassland management practices on available substrate and microbial biomass stoichiometry

Long-term biomass management significantly affected mean C:N_{av} (F = 3.37, P = 0.034) and C:P_{av} ratios (F = 37.84, P < 0.001; Figure 5.2A, B). In particular, mean C:N_{av} ratio for M_fC_1 and M_iC_1 were slightly but significantly lower than those for M_0 by 0.210 (P = 0.019) and 0.269 units (P = 0.003), which was likely related to the N_{av} depletion for the latter (Table 5.3). For M_iC_0, mean C:P_{av} ratio was by 1.39 units (P < 0.001) significantly higher while mean C:N_{av} ratio was similar compared to those for M_0. Neither C:N_{av} or C:P_{av} ratio was significantly different between the N addition treatments.

Although mean values of C_{MB} and N_{MB} were significantly lower for M_0 compared to those for the mown treatments, their ratio (C:N_{MB}) was not significantly different between the biomass and the N addition treatments (Figure 5.2C). Likewise, there was no significant difference in the microbial biomass C:P ratio (C:P_{MB}) between the treatments (Figure 5.2D).

In all treatments, mean values of C:N_{av} and C:P_{av} were much lower than those for C:N_{MB} and C:P_{MB}, respectively, indicating greater microbial C demand relative to those for N and P. This caused an imbalance in C:N and C:P ratios between microbial biomass and available substrates in all treatments (Figure 5.3A, B). While there were no significant differences in C:N imbalance between the treatments, there were significantly lower values for C:P imbalance (i.e., closer to 0) for M_iC_0 (F = 15.29, P < 0.001) compared to the values for the other treatments.

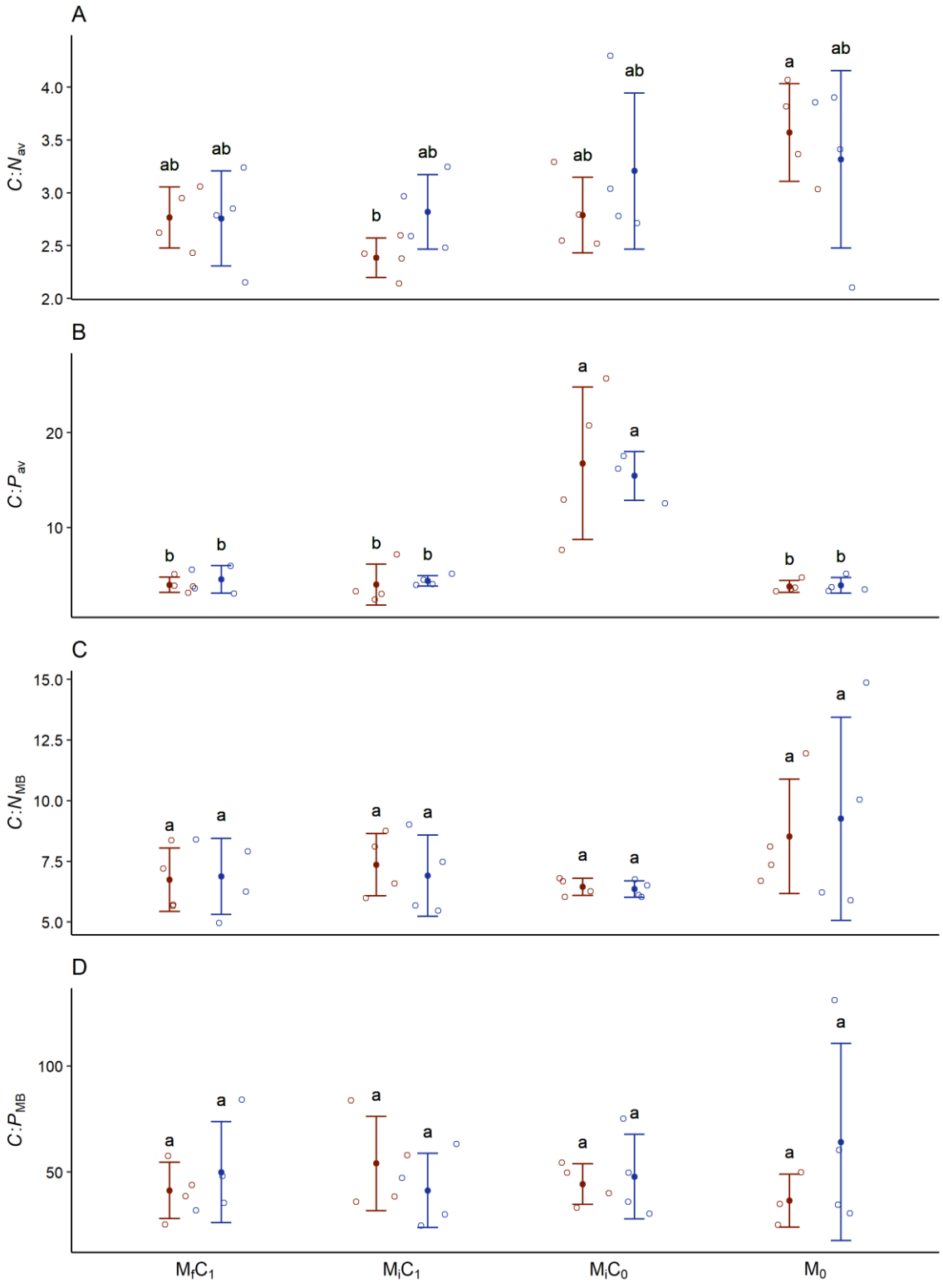

Figure 5.2. Stoichiometric ratios of soil available C:N (C:N_{av}) (A), available C:P (C:P_{av}) (B), microbial biomass C:N (C:N_{MB}) (C), and microbial biomass C:P (C:P_{MB}) (D) in response to biomass management (x-axis, for label identifiers see Table 5.1) with (blue) and without (red) nitrogen addition. Closed points and error bars represent means and standard deviations, respectively. Observations are scattered randomly as open points around the mean. Different letters indicate significant differences among the biomass and N treatments ($P < 0.05$).

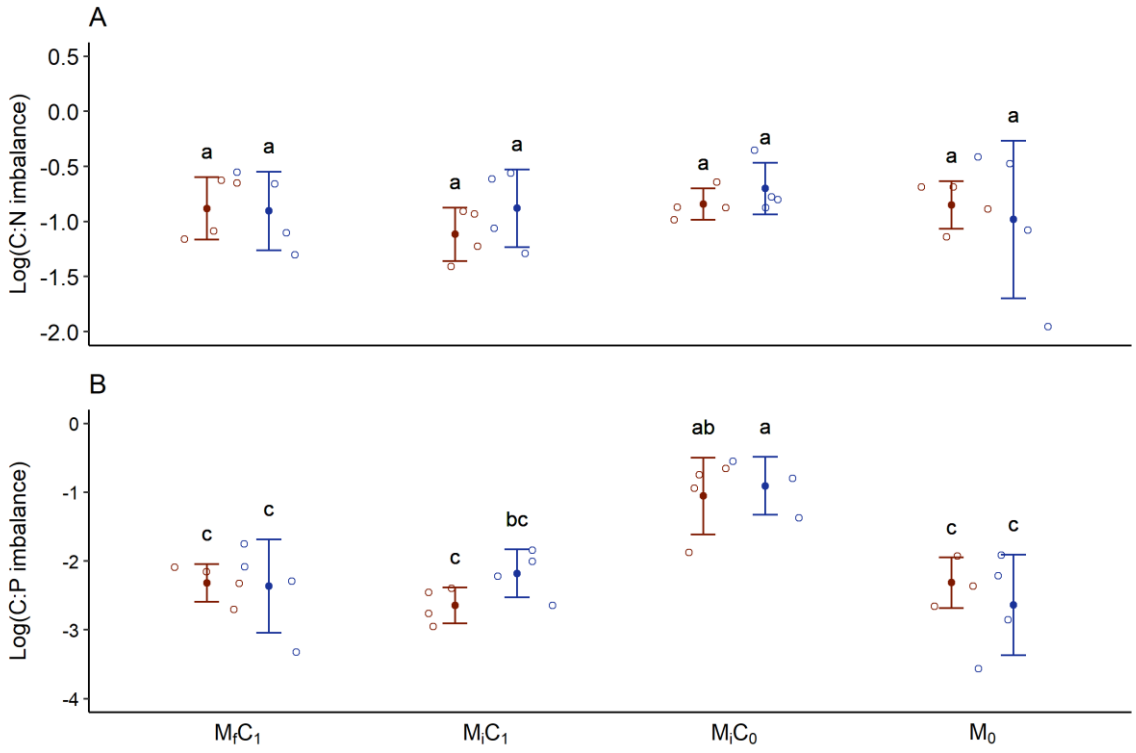

Figure 5.3. Stoichiometric imbalance between microbial biomass and their available substrates for C:N (A) and C:P ratios (B) in response to biomass management (*x*-axis, for label identifiers see Table 5.1) with (blue) and without (red) nitrogen addition. Closed points and error bars represent means and standard deviations, respectively. Observations are scattered randomly as open points around the mean. Different letters indicate significant differences among the biomass and N treatments ($P < 0.05$).

5.4.3 Relationships between available substrate stoichiometry and microbial metabolic elemental limitation

Mean extracellular enzyme activities were consistently higher for M_fC_1 and M_iC_1 in comparison to those for M_0 (Figure A.3.1, Appendix A.3). Vector analysis showed that for M_fC_1 and M_iC_1, mean vector lengths were 13 ($P < 0.001$) and 16% ($P < 0.001$) higher, respectively, of those for M_0, indicating higher metabolic C limitation when clippings were retained ($F = 19.58$, $P = < 0.001$, $R^2 = 0.693$; Figure A.3.2A, Appendix A.3). Furthermore, vector angles were far above 45° in all treatments, indicating strong P co-limitation, with the highest mean values for M_iC_0 (Figures A.3.2B, A.3.3, A.3.4 Appendix A.3). While there were no significant differences in vector length associated with N addition, mean vector angle decreased marginally but significantly by 1.48° in the treatments with added N, suggesting that N addition reduced metabolic P limitation ($F = 5.83$, $P = 0.023$, $R^2 = 0.381$).

The available substrate C:N ratio (C:N_{av}) was linearly related to vector length (SMA, $P < 0.001$, $R^2 = 0.335$; Figure 5.4A). This negative relationship between C:N_{av} and vector length indicates that the activity of C-acquiring enzymes decreased with increasing C availability relative to N availability. Contrary to that, C:P_{av} was not related significantly to vector length (Figure 5.4B). Furthermore, neither C:N_{av} nor C:P_{av} were significantly associated with vector angle (Figure 5.4C, D).

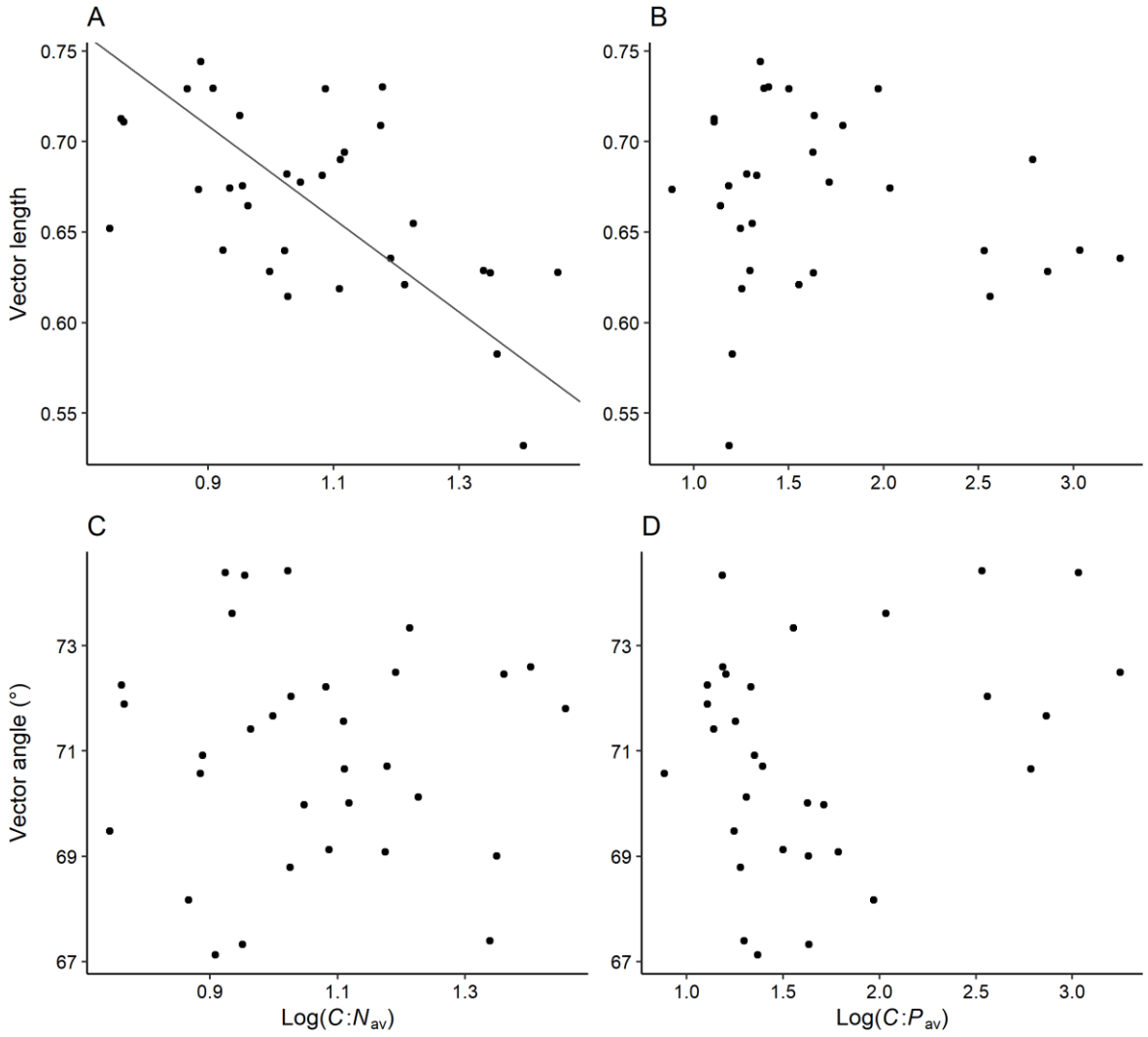

Figure 5.4. Relationships between available substrate C:N ratio (C:N_{av}) (A, C) or C:P ratio (C:P_{av}) (B, D) and vector length (A, B) and vector angle (C, D). The significant linear relationship is indicated by the solid line ($P < 0.05$).

5.4.4 Microbial community composition

There were significant differences in the microbial community composition among the different biomass treatments (Figure A.3.5A; Appendix A.3). PERMANOVA showed that 37.5 % of the variation in the microbial community was explained by the biomass treatments (P = 0.001), while the effects of N addition were not significant (Figure A.3.5B, Appendix A.3). The microbial community for M_0 diverged strongly from those for M_fC_1, M_iC_1, and M_iC_0 (Figure A.3.5A, Appendix A.3). In particular, there was a significantly lower fungal:bacterial ratio for M_0 than that for M_fC_1, M_iC_1, and M_iC_0 (F = 8.64, P < 0.001; Figure 5.5A). Similarly, the gram-positive:gram-negative bacterial ratio was lower for M_0 than that for M_fC_1, M_iC_1, and M_iC_0, although these differences were not significant statistically (Figure 5.5B).

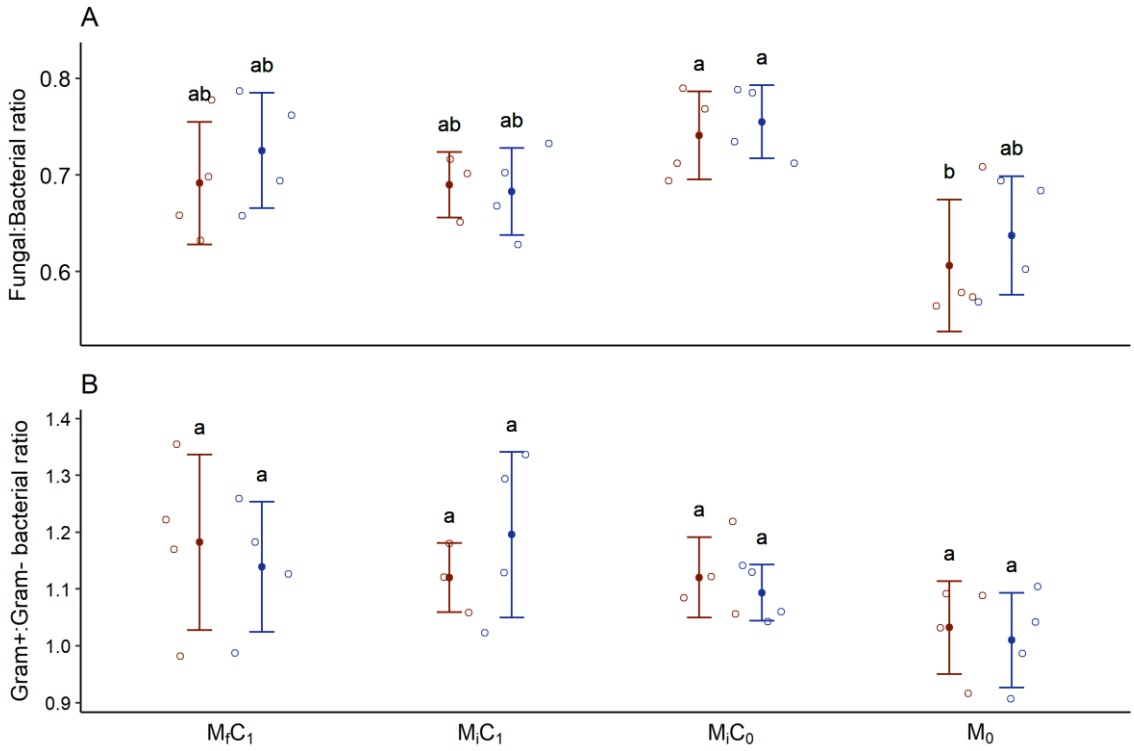

Figure 5.5. Fungal:bacterial (A) and gram-positive:gram-negative bacterial ratios (B) in response to biomass management (x-axis, for label identifiers see Table 5.1) with (blue) and without (red) nitrogen addition. Closed points and error bars represent means and standard deviations, respectively. Observations are scattered randomly as open points around the mean. Different letters indicate significant differences among the biomass and N treatments (P < 0.05).

An optimal NMDS configuration was reached using two dimensions and a final stress value of 0.141. Vector fitting onto the NMDS-ordinated microbial community indicated significant correlations of the microbial community composition with C:N_{MB}, metabolic C limitation, R_{basal}, C_{POM}, and C_{MAOM} (Figure 5.6, Table 5.5). Common to all these vectors in the ordination is some component of C in the system, such as C concentrations in organic matter fractions (C_{POM} and C_{MAOM}), microbial biomass C:N ratio (C:N_{MB}), C respired by heterotrophic microbes (R_{basal}), and metabolic C limitation. This highlights the dependence of the microbial community on C and that the demand for and acquisition of C are distinct for each microbial community.

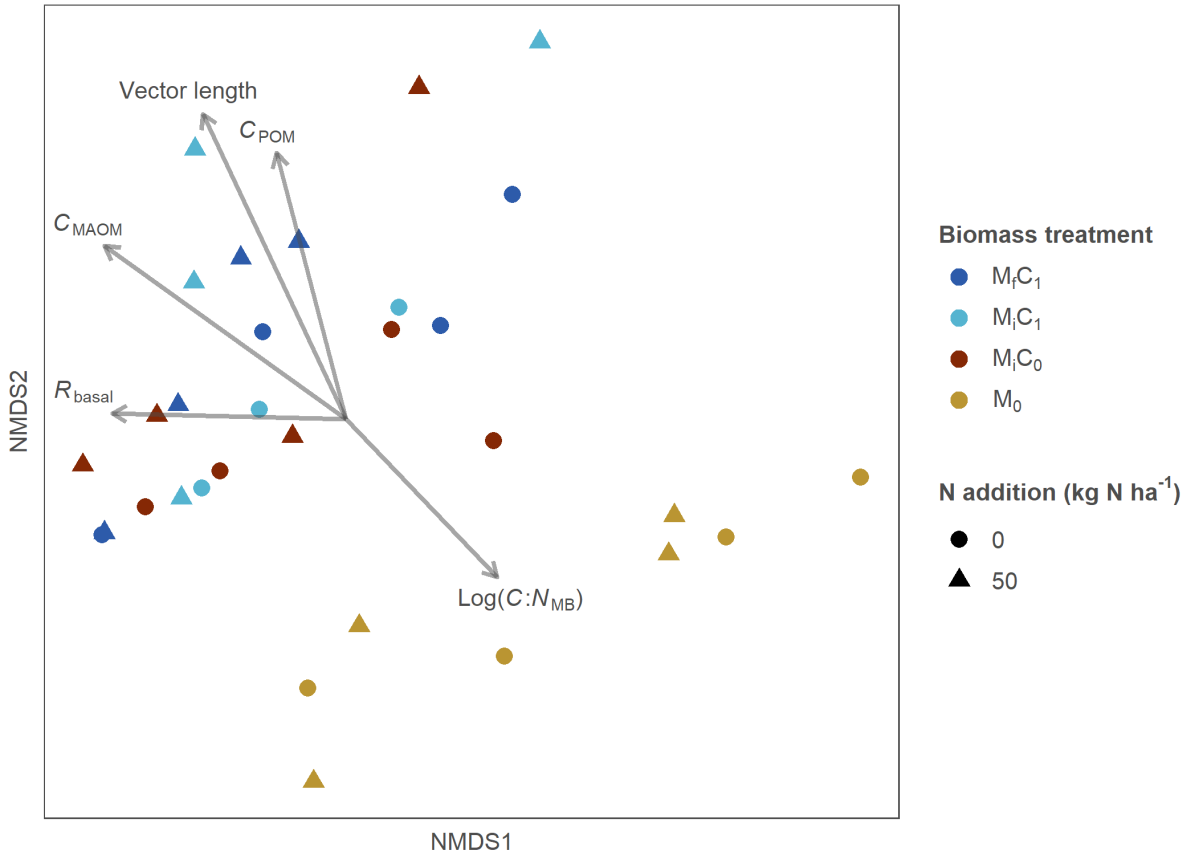

Figure 5.6. Non-metric multidimensional scaling (NMDS) ordination based on distance matrix calculated from relative PLFA biomarker abundances (mol%) of biomass (colours) and nitrogen addition (symbol) treatments. Arrows represent significant vector fits of environmental variables (see Table 5.5 for correlation coefficients and *P*-values).

Table 5.5. Squared Pearson correlation coefficients (R^2) and *P*-values of selected soil properties and functional processes to the NMDS ordinated microbial community data. Non-significant correlations are not shown.

	R^2	*P*
Vector length	0.4176	0.001
C_{MAOM} (g C kg^{-1})	0.3603	0.002
C_{POM} (g C kg^{-1})	0.2705	0.015
R_{basal} (µg CO_2-C g^{-1} h^{-1})	0.2387	0.018
C:N_{MB}*	0.1873	0.046

* log-transformed

5.4.5 Effects of soil microbial community composition and metabolic elemental limitation on soil organic matter fractions

The C and N concentrations of both POM and MAOM fractions in this study were within the range of SOM concentrations in European grasslands (Cotrufo et al., 2019) (Table 5.3, Figure A.3.6A, B, Appendix A.3). Both C_{POM} (F = 8.70, P = 0.006, R^2 = 0.330) and C_{MAOM} (F = 14.21, P < 0.001, R^2 = 0.369) increased with vector length (Figure 5.7A, E), but there was no significant effect of vector angle (Figure 5.7B, F). Although there were significant correlations between the microbial community composition and C_{POM} and C_{MAOM} (see section *5.4.4*), there were no clear relationships between the fungal:bacterial ratio and C_{POM} or C_{MAOM} (Figure 5.7C, G). However, C_{POM} increased significantly with gram-positive:gram-negative bacterial ratio (F = 10.82, P = 0.003, R^2 = 0.279; Figure 5.7D), while there was no significant relationship between C_{MAOM} and gram-positive:gram-negative bacterial ratio (Figure 5.7H).

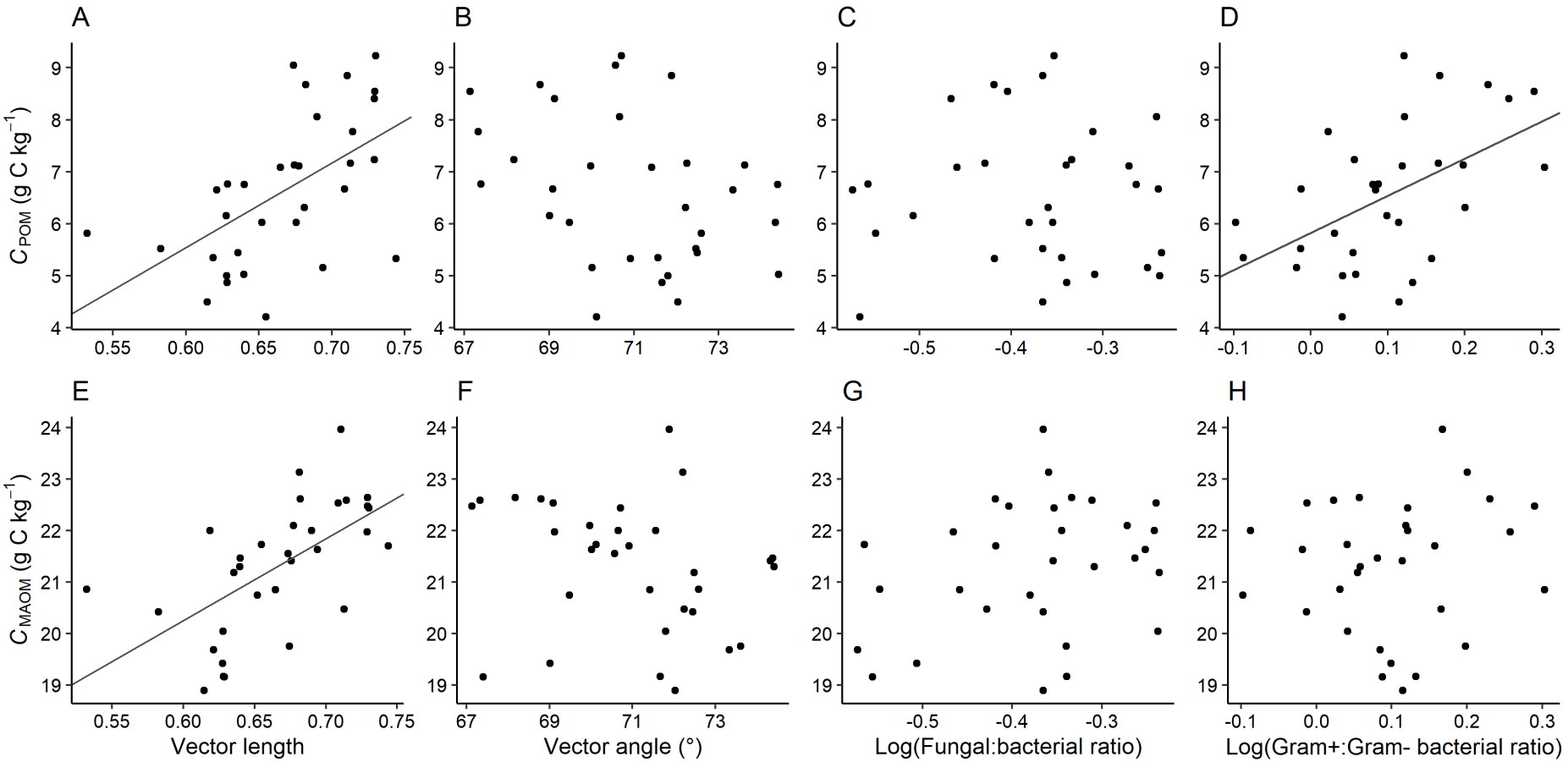

Figure 5.7. Relationships between particulate (C_{POM}) (A-D) or mineral-associated organic matter (C_{MAOM}) (E-H) concentrations and vector length (A, E) and vector angle (B, F), or fungal:bacterial (C, G) or gram-positive:gram-negative bacterial ratio (D, H). Significant linear relationships are indicated by the solid lines ($P < 0.05$).

5.4.6 Effects of microbial community composition and elemental limitation on soil functional processes

Basal respiration rate was related significantly to $C{:}N_{MB}$ (F = 9.08, P = 0.005, R^2 = 0.245; Figure 5.8A), but not with $C{:}P_{MB}$ (Figure 5.8B). In addition to the effect of microbial biomass stoichiometry, R_{basal} was affected by the microbial community composition. The increase in R_{basal} with increased fungal:bacterial ratio (F = 15.74, P < 0.001, R^2 = 0.364; Figure 5.8C) indicated a higher R_{basal} for communities with greater fungal biomass relative to bacterial biomass. Changes in gram-positive:gram-negative bacterial ratio were not related to R_{basal} (Figure 5.8D).

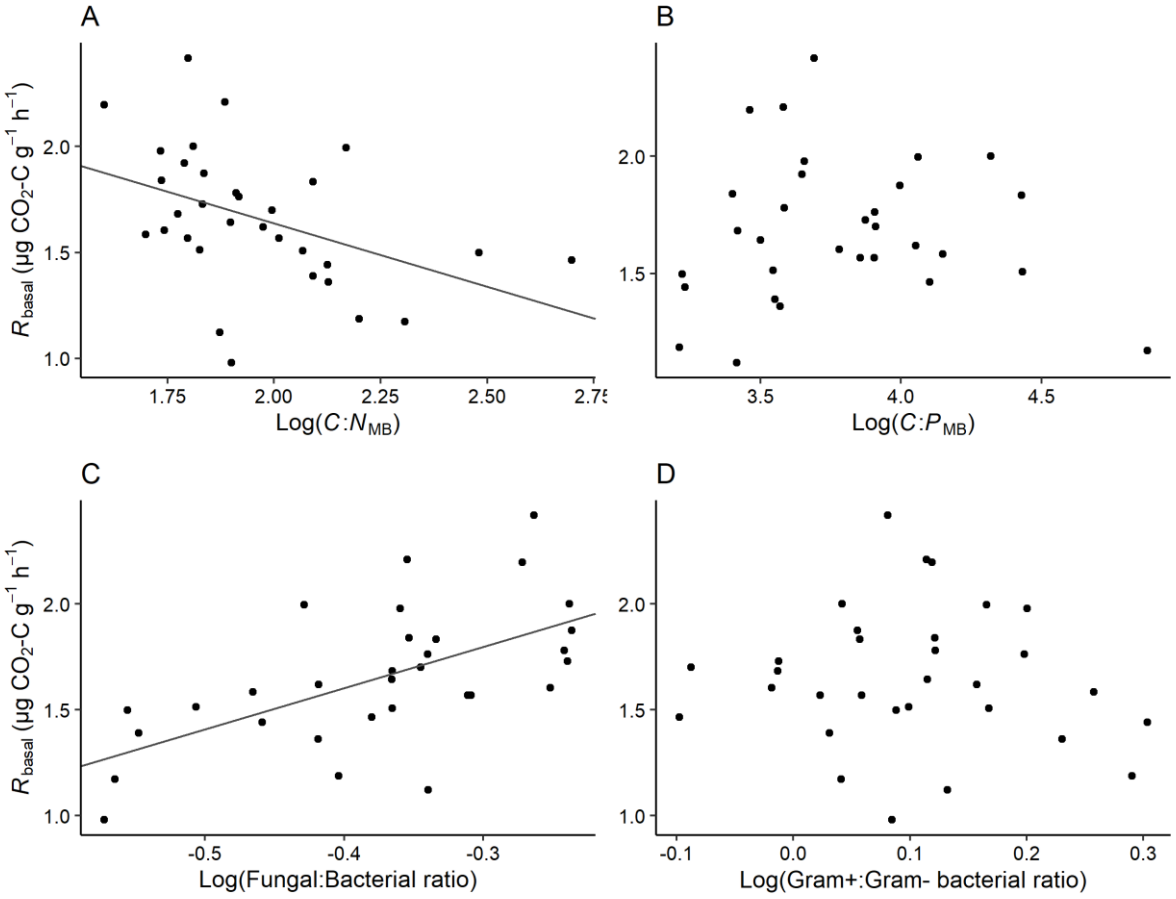

Figure 5.8. Relationships between basal respiration rate (R_{basal}) and microbial biomass C:N ($C{:}N_{MB}$) (A) and C:P ($C{:}P_{MB}$) (B), fungal:bacterial (C) and gram-positive:gram-negative bacterial ratio (D). The significant linear relationship is indicated by the solid lines (P < 0.05).

There were no significant relationships between stoichiometric or microbial community indices and N_{min} (Figure A.3.7, Appendix A.3). Also, there was no significant association between N_{nit} and microbial biomass stoichiometry or microbial community composition (data not shown). Though not statistically different from zero, there was a positive trend in N_{nit} with microbial metabolic C limitation, indicated by vector length (Figure 5.9A), and negative trends with metabolic P limitation, indicated by vector angle (Figure 5.9B), C:N_{av} (Figure 5.9C), and C:P_{av} (Figure 5.9D).

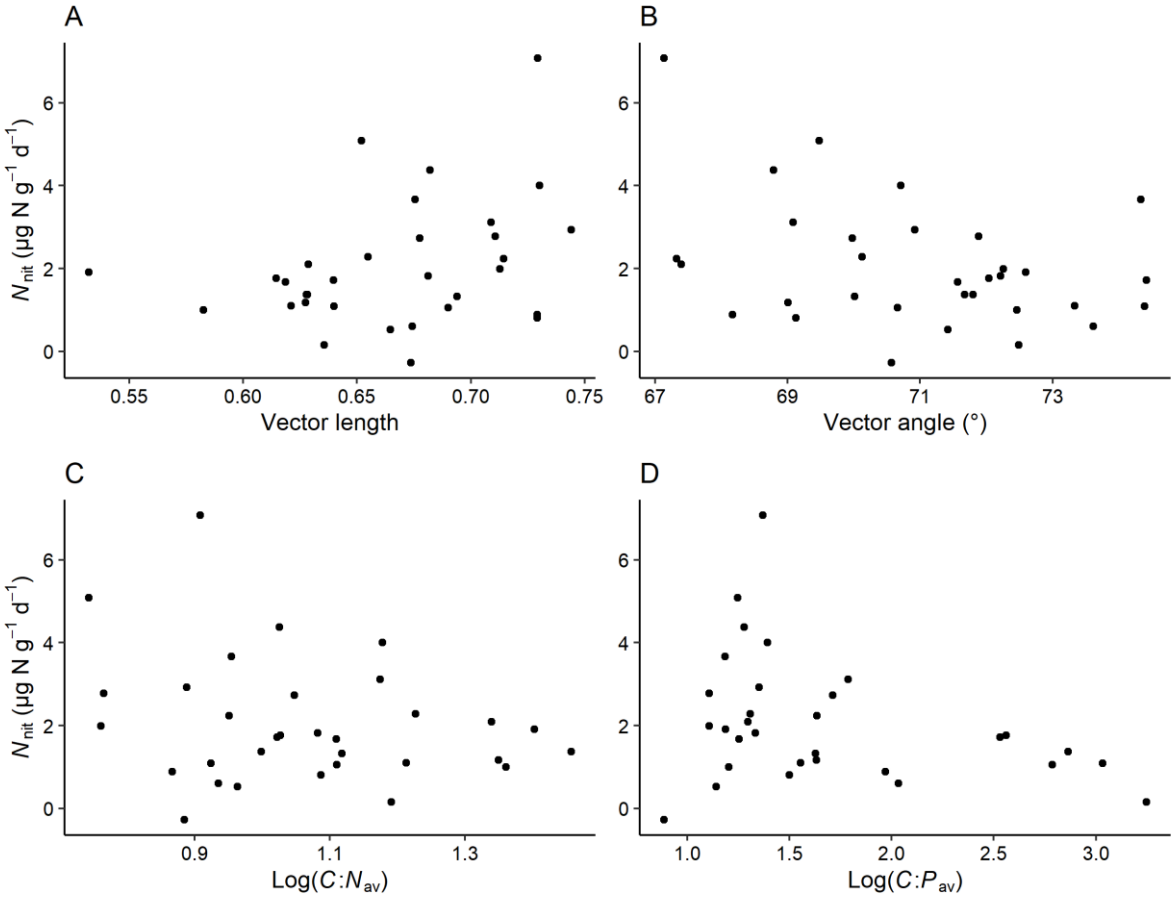

Figure 5.9. Relationships between gross nitrification rate (N_{nit}) and vector length (A) and vector angle (B), available C:N (C:N_{av}) (C) and available C:P ratio (C:P_{av}) (D). None of the relationships were significant.

5.5 Discussion

5.5.1 Long-term grassland management effects on available C:N:P stoichiometry

Different long-term grassland management practices led to strong differences in C:P_{av} but not in C:N_{av} among the treatments, thereby partially supporting the first hypothesis that plant biomass removal and no N addition would lead to distinct C:N:P stoichiometry of available soil substrates.

Long-term retention of plant litter after mowing led to a significant net increase in total soil elemental concentrations (C_t, N_t, and P_t) compared to those for the never mown treatments and treatments where clippings were removed. Although many previous reports on soil stoichiometry and its effect on the soil microbiome have used total elemental ratios (Cleveland and Liptzin, 2007; Fanin et al., 2017; Mooshammer et al., 2014a), available elemental ratios (C:N_{av} and C:P_{av}) were used here because they better reflect the substrates that soil microorganisms depend on and are, therefore, better suited for calculating stoichiometric imbalances (Fanin et al., 2013; Kaiser et al., 2014; Yuan et al., 2019). However, it should be noted that the measurements used in this study consisted of dissolved organic C, dissolved organic and inorganic N, and $NaHCO_3$-extractable inorganic P, which may not be compatible with approaches used in other studies. While the absolute values of the stoichiometric ratios derived here may differ from those used in other studies, they are appropriate to allow comparisons between treatments. Further, previous studies on this field trial have shown that there is very little evidence that labile organic P contributes substantially to short-term microbial P uptake (Boitt et al., 2018a, 2018b). Nevertheless, the measurement and interpretation of the components of dissolved organic elements to derive stoichiometric elemental ratios requires further evaluation.

Mowing significantly affected soil C:N_{av}, with higher C:N_{av} in the never mown treatments than for the mown treatments. This was most likely because of available N depletion, as shown by relatively low values of N_{av} and N_{shoot} in the never mown treatments. Cessation of mowing can reduce N turnover and availability through a change in the plant community composition (Robson et al., 2007). In the long-term trial studied here, there was a shift in plant species composition, with N-fixing clover present in the mown treatments but absent in the never mown treatments (Adair et al., 2013; Dignam et al., 2019), which may explain the observed depletion of available N in the latter.

Significant differences in soil C:P_{av} ratios between the biomass treatments were driven by the marked decrease in P_{av} associated with long-term biomass removal. The fact that removing plant litter depletes soil P_{av} more than soil C_{av} and N_{av} was observed previously and is likely because a significant amount of P_{av} in grassland systems is derived from decomposing plant residues and increasingly limited resupply from more stable P fractions (Boitt et al., 2018a; Farrell et al., 2014; McDowell et al., 2016; Simpson et al., 2012). In contrast, the majority of C_{av} and N_{av} in these soils probably originate from C rhizodeposition, SOM decomposition, N fertiliser addition, and biological N-fixation (Adair et al., 2013; Gardner and Drinkwater, 2009; Jilling et al., 2018; Schimel and Bennett, 2004; Sokol et al., 2019). Thus,

while there is a continuous resupply of C_{av} and N_{av}, resulting in the C:N_{av} ratio remaining relatively constant among the different biomass treatments, long-term removal of plant biomass progressively led to P_{av} depletion, causing significant differences in soil C:P_{av} ratios between the treatments.

5.5.2 Effects of stoichiometric imbalance between available substrates and microbial elemental requirements on the soil microbial community

In partial support of the second hypothesis, there was a stoichiometric imbalance between available substrates and the biomass of the microbial communities, indicating microbial C limitation in all treatments, but there were no differences among the treatments. Microbial C limitation was associated with differences in microbial community composition and extracellular enzyme production.

In all treatments, C:N and C:P ratios were both higher for the soil microbial biomass than those for the available substrates, resulting in significant stoichiometric imbalances between available substrates and microbial biomass. Based on current theory that microbial use of available substrates is optimal when the substrate stoichiometry matches that of its microbial consumers (Buchkowski et al., 2015), the findings indicate that microbial C demand was not satisfied relative to the demands for N and P. This agrees with the widely accepted assumption that soil microbial communities are limited primarily by available C rather than available N or P (Hobbie and Hobbie, 2013; Soong et al., 2020). Microbial C limitation may have been reduced when plant litter removal depleted P_{av} and caused a shift from C to P limitation. However, even under conditions of P_{av} exhaustion, the C:P imbalance indicated that the microbial C demand was higher than that for P. Therefore, it can be assumed that the microbial community was primarily limited by C and that decreasing P_{av} may have led to C and P co-limitation.

The interpretation that the soil microbial community was co-limited by C and P was supported for all treatments from the analysis of eco-enzymatic stoichiometry. These results are consistent with those from previous studies that have shown soil microbial C and P co-limitation in grassland, crop, and forest ecosystems (Chen et al., 2019; Liu et al., 2020; Zheng et al., 2020). Retaining plant biomass after mowing was associated with the highest metabolic C limitation (vector length) compared to that for the other treatments. Hence, it is possible that the microbial production of C-acquiring enzymes was stimulated by high C_t in these treatments, since soil microorganisms release extracellular enzymes only in the presence of complex substrates where decomposition requires enzyme-catalysed reactions (Allison and Vitousek, 2005). Considering both results of eco-enzymatic stoichiometry and stoichiometric imbalances between microbial biomass and available substrates, the findings suggest microbial growth was most limited by C, while co-limitation by P was of secondary importance.

The interdependence between the soil microbial community composition and metabolic C limitation (vector length) supports the hypothesis of a characteristic C demand for each microbial community (Don et al., 2017; Kaiser et al., 2014). Different C requirements between microbial communities can be reflected in changing extracellular enzyme activities, as previously shown for Siberian soils (Schnecker et al., 2015). Changes in extracellular enzyme activities can lead to altered soil substrate availability for microbial uptake, thus serving to alleviate the stoichiometric imbalance between available substrates and microbial biomass (Mooshammer et al., 2014b; Sinsabaugh and Follstad Shah, 2012).

The significant relationship between the microbial community composition and the C:N_{MB} ratio suggests that differences in soil microbial biomass stoichiometry between the mown and never mown treatments were likely due to shifts in the microbial community composition (Fanin et al., 2013; Heuck et al., 2015; Mouginot et al., 2014). The mown treatments were characterised by higher fungal:bacterial ratios and marginally higher gram-positive:gram-negative bacterial ratios compared to the never mown treatments. The higher abundance of fungi relative to bacteria in the mown treatments may be related to their importance in decomposing plant litter and their strong dependence on fresh organic inputs, such as rhizodeposits (Malik et al., 2016; Pausch et al., 2016; Potthoff et al., 2006). Because rhizodeposition can increase to a maximum during active plant growth and declines with increasing plant age (Hütsch et al., 2002; Nguyen, 2003; Pausch and Kuzyakov, 2018), plant regrowth after mowing may promote increased C rhizodeposition (Mawdsley and Bardgett, 1997) that stimulates fungal growth.

5.5.3 Implications for soil organic matter concentrations, basal soil respiration, and N transformations

The findings that microbial C limitation was associated with increasing concentrations of SOM fractions support the third hypothesis. The relationships between C_{POM} and C_{MAOM} and metabolic C limitation (vector length) suggests that the microbial community became increasingly limited by C despite increasing concentrations of POM and MAOM. These findings support the recently emerging concept that SOM formation and decomposition are regulated by microbial accessibility (Cotrufo et al., 2015; Dungait et al., 2012b; Lavallee et al., 2019). An increasing concentration of MAOM may be associated with decreasing microbial access, thereby increasing microbial C limitation. Increased metabolic C limitation was indicated by a proportionally higher C-acquiring enzyme activity, which is consistent with the 'enzyme link' hypothesis proposed by Cenini et al. (2015), where increased microbial C limitation is linked to greater C stabilisation in MAOM via increased production of C-acquiring enzymes. It is hypothesised that increased C-acquiring enzyme activity promotes microbial biomass growth and, subsequently, microbial necromass, and the latter may be adsorbed onto reactive mineral surfaces to form microbially inaccessible MAOM (Cenini et al., 2015; Cotrufo et al., 2013). The positive association between vector length and C_{POM} and C_{MAOM} supports this hypothesis.

The observed relationship of the microbial community composition with C_{POM} and C_{MAOM} adds further support. The microbial community is composed of a variety of microbial taxa which are adapted to localised substrate availability, i.e., different forms of C compounds (Hanson et al., 2008; Kramer and Gleixner, 2008; Schimel and Schaeffer, 2012). The capacity of soil microorganisms to decompose complex soil C compounds is therefore partly dependent on the composition of the microbial community (Don et al., 2017). Gram-negative bacteria are generally associated with simple C compounds and gram-positive bacteria with structurally more complex C compounds (Fanin et al., 2019; Orwin et al., 2018). Because these two bacterial groups utilise different C substrates, their relative abundances could indicate C limitation (Fanin et al., 2019). In the context of this study, the positive relationship between the gram-positive:gram-negative bacterial ratio and C_{POM} may indicate increasing C limitation for microbial activity with increasing C_{POM}.

The microbial community composition and the C:N_{MB} ratio influenced R_{basal} significantly, suggesting that microbial communities with lower C:N_{MB} ratio utilised more C for catabolic respiratory reactions than microbial communities with a higher C:N_{MB} ratio. An inference from the third hypothesis is that, it is possible that microbial communities with lower C:N_{MB} ratios were subjected to stronger C limitation and that their acquired C may have been used predominantly for essential maintenance (i.e., respiration) than for biomass growth (Schimel and Schaeffer, 2012; Schimel and Weintraub, 2003). C:N_{MB} ratios in microbial communities dominated by fungi are typically higher than those in communities dominated by bacteria and this has been associated with slower elemental cycling and lower respiration rates (Malik et al., 2016; Orwin et al., 2016; Six et al., 2006; Wardle, 2004). While this is consistent with the negative correlation between C:N_{MB} and R_{basal} observed here, the negative relationship of fungal:bacterial ratio to R_{basal} is contrary to the hypothesis of slower C cycling with increased fungal abundance. Similar inconclusive results between fungal abundance and R_{basal} have been shown in other studies, suggesting that microbial community composition may not necessarily predict rates of C cycling (Orwin et al., 2020; Rousk and Frey, 2015).

In contrast to the third hypothesis, the results provide no clear evidence for an effect of stoichiometric or community indices on soil functional processes related to N cycling. Both N_{min} and N_{nit} were within the range reported previously for grasslands (Booth et al., 2005). Although statistically not significant, N_{nit} tended to decline with increasing soil C:N_{av}, suggesting that N availability may have limited N_{nit}. It is well known that N availability is the main factor regulating nitrification (Booth et al., 2005; Li et al., 2018) and this is further supported by the observed increase in N_{nit} in the treatments with added N. In contrast to a recent study from Schleuss et al. (2021), who showed that N_{min} was influenced by changing substrate stoichiometry in grassland soils, there was no relationship between N_{min} and any one stoichiometric indicator in this study. The lack of previous studies investigating the impact of substrate and microbial biomass stoichiometries on soil N cycling constrains the interpretation of findings here. Therefore, further research in this direction is needed, especially considering the effect of seasonal variability in microbial elemental limitation on N transformation rates.

5.6 Conclusions

This study has clearly demonstrated that continuous removal or retention of plant biomass after mowing can affect the elemental C:N:P stoichiometry of soil substrates available for microbial uptake. Available inorganic P was strongly depleted by continuous removal of biomass, yet the microbial biomass stoichiometry and the eco-enzymatic stoichiometry suggest that the microbial community was limited primarily by C. In response to imbalanced substrate stoichiometry, there were adjustments in microbial community composition and enzyme production, and this resulted in altered SOM concentrations and basal soil respiration rates. The relationship between microbial metabolic C limitation and SOM concentrations may support the assumption that SOM decomposition is limited by microbial accessibility, leading to increasing microbial C limitation. These findings highlight the importance of stoichiometric regulation for microbially mediated soil processes and could have important implications for SOM concentrations and soil C dynamics under different grassland management practices. The lack of a clear relationship of stoichiometric and microbial indices on soil N mineralisation and nitrification, in addition to the limited number of previous studies, emphasises the need for further research to elucidate the role of microbial elemental limitation on soil N cycling.

Chapter 6
Synthesis and conclusions

6.1 Overview

This research investigated the biogeochemical coupling between C and N cycles in grassland systems under different management practices in laboratory and field conditions. The overall objective was to investigate the biogeochemical coupling of soil C and N cycles in grassland systems. The effects of increased C and N availabilities on soil C and N cycling was tested under controlled conditions in the laboratory. This was followed by a field study, where the influences of biomass removal by mowing, plant litter retention, and N fertiliser addition on soil C and N cycling were investigated.

At the outset of this thesis, three specific objectives were established and the key findings from experimental work related to each objective were:

Objective 1: Identify the effects of increased available C supply from plant roots on the regulation of nitrification for different plant species (Chapter 3).

Key findings:

a. Increasing N availability was associated with increased C rhizodeposition.
b. Increased C rhizodeposition led to decreased soil nitrification in the root zone.

Objective 2: Determine the effects of increasing N availability on ecosystem C balance, C rhizodeposition, and regulation of C and N cycling by the microbial community under two grassland plant species (Chapter 4).

Key findings:

c. Net ecosystem photosynthesis, plant N uptake, and C rhizodeposition were greater for *Plantago lanceolata* than for *Lolium perenne*, resulting in overall higher net ecosystem CO_2 uptake for the former.
d. N availability affected plant species-specific allocation of rhizodeposited C to different microbial groups.

Objective 3: Determine the relationships between microbial C and nutrient requirements, substrate stoichiometry, and the microbial community composition under different grassland management practices (Chapter 5).

Key findings:

e. The microbial community was strongly dependent on C supply and the microbial C demand was distinct for each microbial community.
f. Microbial communities limited by C availability were associated with greater concentrations of SOM fractions.

6.2 Synthesis of key findings

6.2.1 Effects of increased C availability on soil nitrification (Chapter 3)

The research in Chapter 3 addressed the objective of investigating the effect of root-derived available C (increased by N addition) on the regulation of soil nitrification activities for different plant species. For this study, a short-term microcosm experiment was set up under controlled conditions with five grassland species treated with low and high rates of N fertiliser. The findings showed clearly that N addition resulted in an increase in water-extractable C concentrations in the planted microcosms but not in the unplanted microcosms, suggesting that the increases in water-extractable C concentrations in the high N treatments were derived from plant roots. The association between increased water-extractable C concentrations and decreased potential nitrification activities suggests that the increase in available C concentration may have stimulated heterotrophic N immobilisation, leading to reduced nitrification.

These findings highlight that root-derived available C concentrations can be manipulated by N addition and support the concept of tight coupling between root-derived available C and soil N cycling through microbial activity in the vicinity of roots (Paterson, 2003). Consistent with previous studies (Groffman et al., 1996; Le Roux et al., 2003; Stienstra et al., 1994), there were no clear differences in the response between plant species. These new insights can contribute to identifying grassland management practices that improve N retention and reduce the risk of N losses from grassland soils. For example, the findings indicate that the risk of N leaching was greatest in the unplanted control soil and when the root-derived available C supply to soil microorganisms was low. Because these conditions are likely to occur in winter or after biomass harvest when photosynthetic activity is reduced, the maintenance of a continuous plant cover and active growth is crucial for increasing N retention by plant N uptake and C rhizodeposition (Malcolm et al., 2014).

The findings from this study raise the question of whether C rhizodeposition increases proportionally to the rate of N added. Also, it was beyond this specific study to investigate the pathways of C cycling through the plant-soil-microbe system. Therefore, the study presented in Chapter 4 was designed to address these research gaps.

6.2.2 Effects of increased N availability on C transfer through the plant-soil-microbe system and regulation of soil microbial C and N cycling (Chapter 4)

The objective in Chapter 4 was to investigate the effects of increasing N addition on net ecosystem C balance, C rhizodeposition, and the regulation of soil functional processes by the microbial community. Two grassland plant species were grown in microcosms under controlled conditions for seven to eight weeks and treated with an increasing addition of N. Measurements of net ecosystem C balance were combined with a $^{13}CO_2$ pulse-labelling experiment to determine the allocation of C in the plant-soil-microbe system. In addition, gross N transformation rates were measured using a ^{15}N isotope pool dilution approach. Plant C uptake and C rhizodeposition increased with increasing N addition and were overall higher for *Plantago lanceolata* than those for *Lolium perenne*. Allocation of rhizodeposited C associated with the different plant species to the different microbial groups was modified by increasing N addition, suggesting that N availability influences microbial processing of rhizodeposited C from different plant species. While microbial uptake of rhizodeposited C was associated with basal soil respiration rate, the associations with N mineralisation and nitrification rates were unclear, which is interpreted as a decoupling of soil C and N cycles with increasing N addition.

The innovative contribution of this study was the use of $^{13}CO_2$ pulse-labelling to trace C through the plant roots and its transfer to the microbial community. This work has provided new insights to the allocation of recently photo-assimilated C to plant, soil, and microbial components. The findings confirmed the usefulness of the combination of measurements of net ecosystem C balance and ^{15}N pool dilution with $^{13}CO_2$ pulse-labelling to trace C through the plant-soil-microbe system and to link the processes involved in C and N cycling. The findings corroborated those from Chapter 3 that C rhizodeposition increased with increasing N availability and that rhizodeposited soil C concentrations under *P. lanceolata* increased more than those for *L. perenne*. The findings provide further support for the concept that plant C uptake and C rhizodeposition can increase proportionally in response to an increasing rate of N addition (here, up to a maximum of 750 kg N ha^{-1}) and that rhizodeposited C is rapidly (hours) transferred to the soil microbial community (Bahn et al., 2013; De Deyn et al., 2011; Denef et al., 2009; Werth and Kuzyakov, 2008). Contrary to the findings from Chapter 3, there was no significant relationship between rhizodeposited C and soil N transformations, which is likely related to the different experimental and analytical methods used. For example, it is possible that the difference could be attributable to the size of the microcosms used. The much smaller size of the microcosms used in Chapter 3 could have led to the soil being more strongly influenced by the plant roots, compared with conditions in the larger microcosms used in Chapter 4. Therefore, it is possible that the coupling between rhizodeposited C and soil nitrification is constrained to the proximity of soil to roots. Further, estimates of soil nitrification from potential nitrification rates (potential nitrification activity) in Chapter 3 and gross nitrification rates (^{15}N pool dilution) in Chapter 4 used different methods that typically lead to very different results (Booth et al., 2005; Hart et al., 1994b; Verchot et al., 2001). Therefore, the results from the studies in Chapter 3 and 4 may not be comparable.

This study discovered that photosynthesis, plant N uptake, and C rhizodeposition during the labelling experiment were higher for *P. lanceolata* than for *L. perenne*, leading to overall higher net ecosystem CO_2 uptake. From these findings it could be inferred that soil C and N cycles were more tightly coupled for *P. lanceolata* than those for *L. perenne*, which might explain the reduced risk for N losses from *P. lanceolata* compared to *L. perenne* observed in previous studies (Carlton et al., 2019; Luo et al., 2018). This is a cautious interpretation and further research is needed because this study was limited by a lack of a significant relationship between microbial utilisation of rhizodeposited C and soil processes regulating N cycling. Yet, the lack of a relationship may suggest a decoupling of C and N cycles with increasing N addition, which is consistent with the hypothesis of Soussana and Lemaire (2014) that excessive N inputs can increasingly decouple soil C and N cycles.

While previous studies have shown that the quantity of rhizodeposited C allocated to different microbial groups is dependent on plant species (Ladygina and Hedlund, 2010; Ngosong et al., 2011), the new contribution from this study is evidence that plant species-specific variation in rhizodeposited C uptake by different microbial groups is dependent on N availability. Thus, plant species-specific effects on C rhizodeposition and microbial uptake of rhizodeposited C observed in low N input systems are likely different from those in high N input systems. These findings will have important implications for soil C cycling in high N grasslands and they highlight the need for future studies to incorporate the interaction between plant species and soil N status in C allocation to the soil microbial community (Fanin et al., 2019; Ladygina and Hedlund, 2010; Malik et al., 2016; Six et al., 2006; Wardle, 2004).

The findings provided evidence that the soil microbial community composition plays an important role in soil functioning (Ernakovich et al., 2021; Nemergut et al., 2014; Schimel and Schaeffer, 2012; Six et al., 2006; Strickland et al., 2009; Wardle, 2004). However, the underlying mechanisms are unclear (Krause et al., 2014). For example, it is possible that a change in microbial community composition contributes to a decoupling of C and N cycles due to different C and N requirements by varying microbial communities (Cleveland and Liptzin, 2007; Mouginot et al., 2014). The identification of such mechanisms regulated by the soil microbial community composition is important for explaining interactions between microbial functional groups and for predictions of ecosystem functioning (Kaiser et al., 2014). This study has provided a framework for further research to address relationships between microbial community composition and function.

The $^{13}CO_2$ pulse-labelling approach is limited to the C allocation dynamics within the soil-plant-microbe system at the specific growth stage of the plant and, therefore, is not able to reflect the whole growth period (Kuzyakov and Domanski, 2000). Furthermore, $^{13}CO_2$ pulse-labelling cannot quantify C fluxes, because C assimilated by the plant is not allocated uniformly but directed towards active growth zones (Paterson et al., 2009). However, for the purposes of analysing the allocation of recently assimilated C within the soil-plant-microbe system, including the identification of primary microbial consumers of rhizodeposited C and their contribution to soil respiration, $^{13}CO_2$ pulse-labelling was shown to be an

appropriate method (Liu et al., 2019; Paterson et al., 2009; Pausch and Kuzyakov, 2018). The use of continuous labelling with $^{13}CO_2$ where there is a continuous supply of the label to the plant, ideally during the entire growth period, can provide estimates of C rhizodeposition rates (Kuzyakov and Domanski, 2000; Paterson et al., 2009).

Because the findings presented in Chapters 3 and 4 were derived from relatively short-term experiments of seven to nine weeks in controlled conditions, they are limited to the effects of the respective treatments on relatively young plants, and they may not be transferrable to older plants and/or species-specific effects in field conditions. Thus, the question arises of whether the mechanistic understanding gained through lab-based experiments can be applied to field conditions, where C and/or N availability vary with different grassland management practices. This was addressed in Chapter 5.

6.2.3 Effects of microbial C and nutrient limitation on soil organic matter fractions and soil microbial C and N cycling (Chapter 5)

The objective of Chapter 5 was to address the question of how microbial communities limited by available C, N, or P affect concentrations of SOM fractions, rates of soil respiration and N transformations. Plant and soil samples were collected from a temperate grassland field experiment that was established 25 years prior to this study and is characterised by consistent and contrasting management practices, comprising biomass removal and N addition treatments. The findings showed that continuous removal of plant litter resulted in P depletion, leading to differences in the stoichiometry of available soil C:N:P among the biomass removal treatments. The stoichiometric imbalance between microbial demand and available substrates suggests that the microbial community was primarily C-limited, which led to a change in the microbial community composition and extracellular enzyme production. The relationship between microbial community composition and concentrations of SOM fractions, soil respiration rate, and C-acquiring enzyme activity suggests that the microbial community is highly dependent on C. While differences in the microbial community composition and metabolic C demand were associated with different soil respiration rates and concentrations of SOM fractions, there were no clear relationships between stoichiometric or microbial indices and soil N transformation rates.

Accounting for stoichiometric regulation of microbial elemental cycling is a critical framework for improving sustainable grassland management practices (Bertrand et al., 2019). The major contribution of this study is demonstration of linkages between microbial elemental requirements and soil biogeochemical processes through the framework of ecological stoichiometry. The findings are consistent with those from previous studies on the same field experiment that continuous removal of plant biomass depletes soil available inorganic P concentrations because of limited resupply of P from sources other than decomposing plant litter (Boitt et al., 2018a; Farrell et al., 2014; McDowell et al., 2016; Simpson et al., 2012). Furthermore, the findings support the assumption that the soil microbial

community is generally limited by available C and not by available N or P (Hobbie and Hobbie, 2013; Soong et al., 2020). The significant relationships between microbial community composition and concentrations of SOM fractions, soil respiration rate, and C-acquiring enzyme activity highlights the interdependence between the microbial community composition and C supply. This emphasises that the requirement for and acquisition of C can vary between different microbial communities (Don et al., 2017). While previous analyses of ecosystem responses to environmental changes have often overlooked the effect of microbial C limitation (Soong et al., 2020), this study has shown that microbial C limitation affects soil C cycling by influencing concentrations of SOM fractions and soil respiration rates.

The relationship between microbial metabolic C limitation and concentrations of SOM fractions may be consistent with recent concepts that SOM formation and decomposition are regulated by microbial access (Cotrufo et al., 2015; Dungait et al., 2012b; Lavallee et al., 2019). This work provides new evidence that stoichiometric imbalance between microbial elemental demand and available substrates leads to increased concentrations of SOM fractions. The findings have important implications for the regulation of SOM concentrations in grassland systems that could lead to improvements in models to predict differences in soil C cycling in relation to different grassland management practices (Soong et al., 2020). For example, in this study, occasional mowing with clippings retained on the soil was associated with increased SOM concentrations, which could lead to increases in SOC stocks and greater N and P retention (Cotrufo et al., 2019; de Vries and Bardgett, 2012).

The lack of relationships between microbial community composition or stoichiometric elemental demand and N transformation rates are consistent with the findings from Chapter 4. While this may suggest a decoupling of C and N cycles with excessive N supply in Chapter 4, the results reported in for Chapter 5 (where N was not in excess) could indicate that the strong C limitation dominated the biogeochemical coupling between C and N. This is explained by the strong limitation of the microbial community by C, resulting in little influence of microbial C utilisation on soil N cycling. This is also consistent with the results reported in Chapter 3, where increased concentrations of available C for microbial uptake was thought to alleviate C limitation and significantly affect nitrification. The opposite effect may have occurred to explain the findings in Chapter 5, where strong C limitation constrained microbial growth, leading to a low stoichiometric N demand of the microbial community. These findings are not consistent with those of Schleuss et al. (2021), who showed that N mineralisation rates in grassland soils were largely regulated by differences in substrate stoichiometry. However, conditions in the grassland systems studied here were markedly different to those for the grassland systems investigated by Schleuss et al. (2021), which were located in Central Europe, South Africa, and in the USA with no mowing treatments, and N and P fertilisers were added annually at a rate 100 kg ha^{-1} for 7 years prior to their study. Therefore, comparing between the findings from this study and those from Schleuss et al. (2021) is difficult. A lack of other previous studies using ecological stoichiometry to link soil biogeochemistry with microbial elemental cycling limited the interpretation of the findings of this study in a broader context and emphasises the need for further research is needed.

In Chapters 4 and 5, the PLFA method was used to analyse the biomass and/or the composition of soil microbial communities by assuming that individual PLFAs can indicate specific microbial groups. However, many PLFAs are not unique to specific microbial taxa and groups (Frostegård et al., 2011; Ruess and Chamberlain, 2010). For example, although the PLFAs 18:2ω6,9 and 18:1ω9 occur in many eukaryotic organisms, including plants, they were designated here as fungal biomarkers, because they were shown to be good indicators for fungi, especially in sieved soils as used in the studies presented here (Frostegård et al., 2011; Kaiser et al., 2010). Nevertheless, considering the uncertainty of using PLFA as biomarkers for microbial groups, PLFA results need to be interpreted carefully. The PLFA method was chosen because it is a rapid and inexpensive approach that can provide a proxy of the microbial community composition with sufficient taxonomic resolution needed for these studies (Frostegård et al., 2011). It has been shown that the PLFA method can provide information that is broadly similar to that from gene metabarcoding techniques and may even be more sensitive in detecting compositional changes in microbial communities (Frostegård et al., 2011; Orwin et al., 2018; Ramsey et al., 2006).

6.3 Conclusions

Through the body of work presented here, it was demonstrated that the coupled biogeochemical cycles of C and N in grassland soils are dependent on stoichiometric elemental requirements of the soil microbial community and that these are strongly influenced by management practices. Increased concentrations of rhizodeposited C available for microbial uptake alleviated C-limiting conditions in close proximity to roots and led to a significant decrease in nitrification. This relationship between N transformation and microbial processing of rhizodeposited C was not evident when N was added in excess of plant uptake, suggesting a decoupling of C and N cycles in high N systems. Because the soil microbial community is generally not limited by available N but by available C (Soong et al., 2020), an increase in C availability is required to increase the stoichiometric N demand of the microbial community and to affect microbial N transformations. This mechanistic concept developed through the laboratory studies was supported by the findings from the field study, where the microbial community was primarily limited by available C and this could have led to the minor observed influence of microbial and stoichiometric indices on N cycling.

This combination of experimental laboratory and field work has emphasised the importance of the tight coupling between soil C and N biogeochemical cycles for regulating ecosystem functions of grassland systems. The findings highlight that a thorough understanding of the mechanisms that couple and decouple C and N cycles is critical to mitigate environmentally damaging effects resulting from uncontrolled decoupling of elemental cycles (Rumpel and Chabbi, 2019). Further, this work has provided consistent evidence for the major role of the microbial community composition in regulating soil elemental cycles and ecosystem functioning, although the underlying mechanisms remain unknown. Better understanding of these mechanisms is needed to inform predictions on ecosystem functioning under different management practices to identify best practice strategies to reduce C and N losses from grassland systems (Bertrand et al., 2019; Kaiser et al., 2014; Macdonald et al., 2018). Research to identify the mechanisms that regulate the function of the soil microbial community and the consequences for biogeochemical processes is still in its infancy and, with the development of new techniques, further progress is expected to occur rapidly in the next decade.

6.3.1 Recommendations for future research

The findings from this PhD research programme raise further questions that could be addressed in future studies to gain a better understanding of the mechanisms regulating the coupling of C and N cycling in differently managed grassland systems. Key areas for future research include the following:

1. The effects of plant age and plant species mixtures on the coupling of C and N cycles are uncertain and require further investigation on monocultures and mixed species swards to reveal potential plant species-specific effects that may change with increasing plant age or through interactions with other species.

2. The influence of the composition of root exudates on soil N cycling should be investigated in future research to differentiate between the effects of root exudate quantity and quality.

3. The specific mechanisms by which the cycling of added N is regulated in the plant-soil-microbe system are unclear. The relative importance of the different pathways of soil N transformation will have consequences for soil N retention and plant and microbial N acquisition. Of particular interest would be to measure effects of N addition on soil organic N concentrations using ^{15}N isotope tracers to chase the added N through the plant-soil-microbe system.

4. More studies integrating the concept of ecological stoichiometry for investigating the influence of microbial elemental requirements and limitations on soil nutrient fluxes and transformation rates are needed, especially in the context of developing agricultural management practices.

5. The influence of the composition of specific microbial groups or taxa and their function in regulating biogeochemical processes is largely unknown and needs the development of new techniques and further research. Microbial network analysis could help identify keystone taxa within microbial communities and their ecological interactions.

6. The findings from this research suggest that there is an interaction between the soil microbial community composition and soil elemental cycling, and that this interaction could be affected by management practices, but the mechanisms are not known. Future studies are needed to relate microbial communities and their activities to soil functional processes, especially in managed grassland systems.

7. The interaction between the soil microbial community composition and soil elemental cycling is likely dependent on plant species. Studies incorporating root functional traits and their effects on the soil microbial community composition and concurrent soil functional processes will improve current understanding of ecosystem function and plant-soil interactions that will lead to improved environmental outcomes.

Appendix A

Supplementary Material

A.1 Supplementary Material for Chapter 3

Table A.1.1. Primers and thermocycling conditions used for real-time qPCR of the targeted functional genes.

Target gene	Primer	Sequence (5'-3')	Reference	Thermocycling conditions	No. of cycles
AOB *amoA*	amoA-1F	GGGGHTTYTACTGGTGGT	(Stephen et al., 1999)	95 °C – 120 s	1
	amoA R-i	CCCCTCNGNAAANCCTTCTTC	(Hornek et al., 2006)	95 °C – 20 s/57 °C – 30 s/72 °C – 30 s/85 °C – 15 s	40
AOA *amoA*	Arch-amoAF	STAATGGTCTGGCTTAGACG	(Francis et al., 2005)	95 °C – 120 s	1
	Arch-amoAR	GCGGCCATCCATCTGTATGT		95 °C – 20 s/55 °C – 20 s/72 °C – 30s/80 °C – 15 s	40

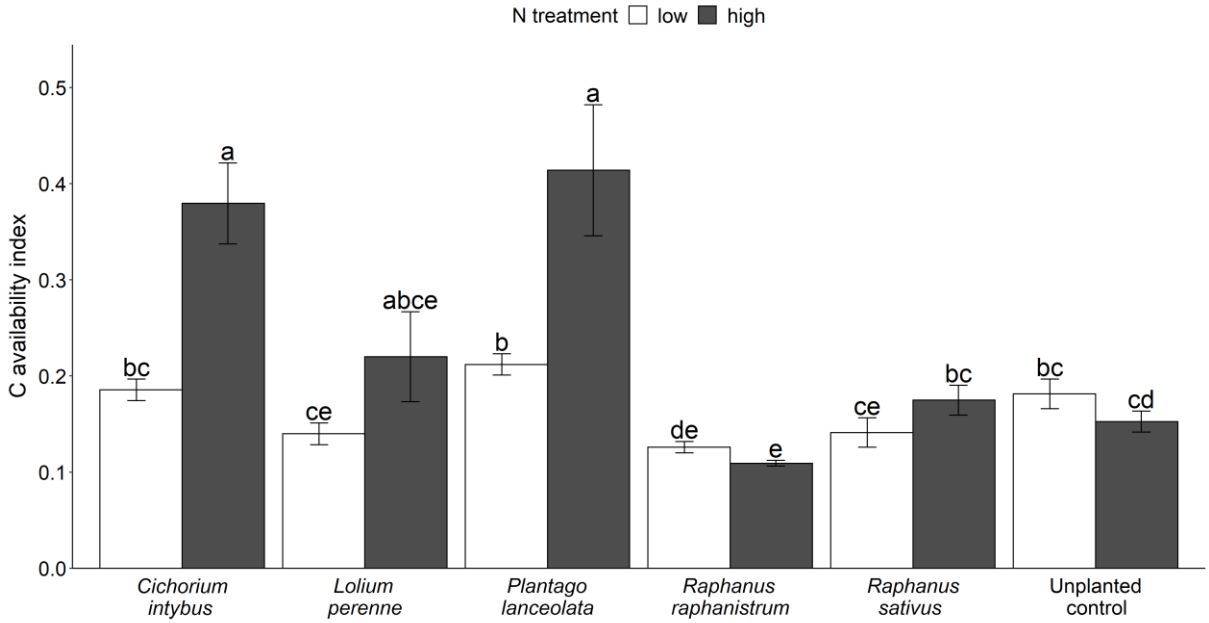

Figure A.1.1. Mean C availability index in the soils under different plant species and N treatments. Error bars represent standard errors, n = 4. Different letters indicate significant differences among plant species or controls and N treatments ($P < 0.05$).

A.2 Supplementary Material for Chapter 4

Table A.2.1. Initial soil properties determined after soil collection.

Soil property	Unit	Value
pH ($CaCl_2$)		5.1
Organic matter	mg kg^{-1}	42.0
Ammonium-N	mg kg^{-1}	4
Nitrate-N	mg kg^{-1}	9
Olsen Phosphorus	mg L^{-1}	16
Potassium	mg kg^{-1}	164
Calcium	mg kg^{-1}	1200
Magnesium	mg kg^{-1}	61
Sodium	mg kg^{-1}	36.8
Sulphate-Sulphur	mg kg^{-1}	6
Cation exchange capacity	me/100 g	12
Base saturation	%	61

Table A.2.2. Model summary for the linear mixed effects model fits for the nitrogen addition (kg N ha^{-1}) and species treatment effects on net ecosystem CO_2 exchange, F_N, and its components, photosynthesis rate, A, soil respiration rate, R_s, and plant respiration rate, R_p. The values for the species effect (*Species*) refers to the effect for *P. lanceolata* relative to *L. perenne*.

F_N (g C m^{-2} h^{-1})			
Fixed effects			
Term	Estimate	95% CI	
Intercept	0.472	0.406	0.538
N_{rate}	-1.3×10^{-4}	-2.1×10^{-4}	-4.7×10^{-5}
Species	4.8×10^{-2}	1.5×10^{-2}	8.0×10^{-2}
Random effects			
Group	Term	Std. Dev.	Variance proportion (%)
Date	Intercept	3.7×10^{-2}	17.8
Residual		7.9×10^{-2}	82.2
A (g C m^{-2} h^{-1})			
Fixed effects			
Term	Estimate	95% CI	
Intercept	1.08	1.12	1.14
N_{rate}	1.2×10^{-4}	-5.5×10^{-6}	2.5×10^{-4}
Species	0.177	0.124	0.230
Random effects			
Group	Term	Std. Dev.	Variance proportion (%)
Date	Intercept	0.00	0
Residual		1.7×10^{-2}	100
R_s (g C m^{-2} h^{-1})			
Fixed effects			
Term	Estimate	95% CI	
Intercept	0.465	0.400	0.531
N_{rate}	8.1×10^{-5}	-6.1×10^{-6}	1.7×10^{-4}
Species	-4.5×10^{-2}	-8.1×10^{-2}	9.2×10^{-3}
Random effects			
Group	Term	Std. Dev.	Variance proportion (%)
Date	Intercept	1.6×10^{-3}	17.4
Residual		7.7×10^{-3}	82.6
R_p (g C m^{-2} h^{-1})			
Fixed effects			
Term	Estimate	95% CI	
Intercept	0.141	6.9×10^{-2}	0.212
N_{rate}	1.6×10^{-4}	4.8×10^{-5}	2.8×10^{-4}
Species	0.172	0.125	0.219
Random effects			
Group	Term	Std. Dev.	Variance proportion (%)
Date	Intercept	1.4×10^{-3}	9.4
Residual		1.3×10^{-2}	90.6

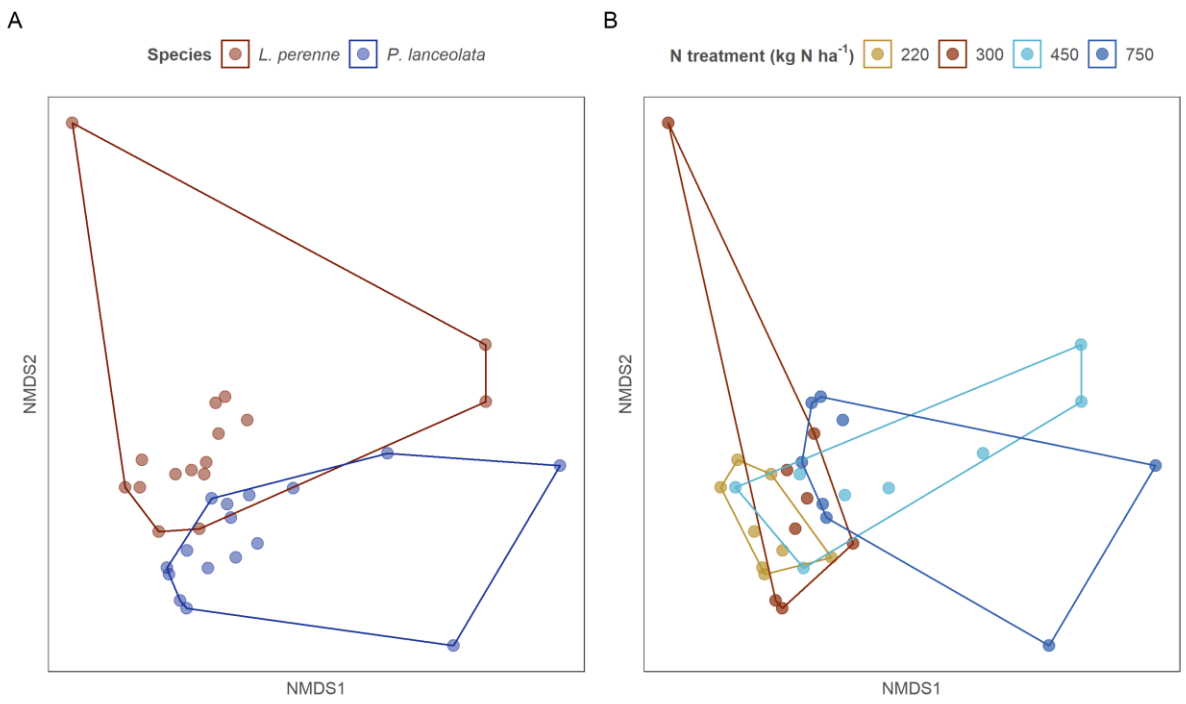

Figure A.2.1. NMDS ordinated soil microbial community composition affected by plant species (A) and by nitrogen addition treatment (kg N ha^{-1}) (B).

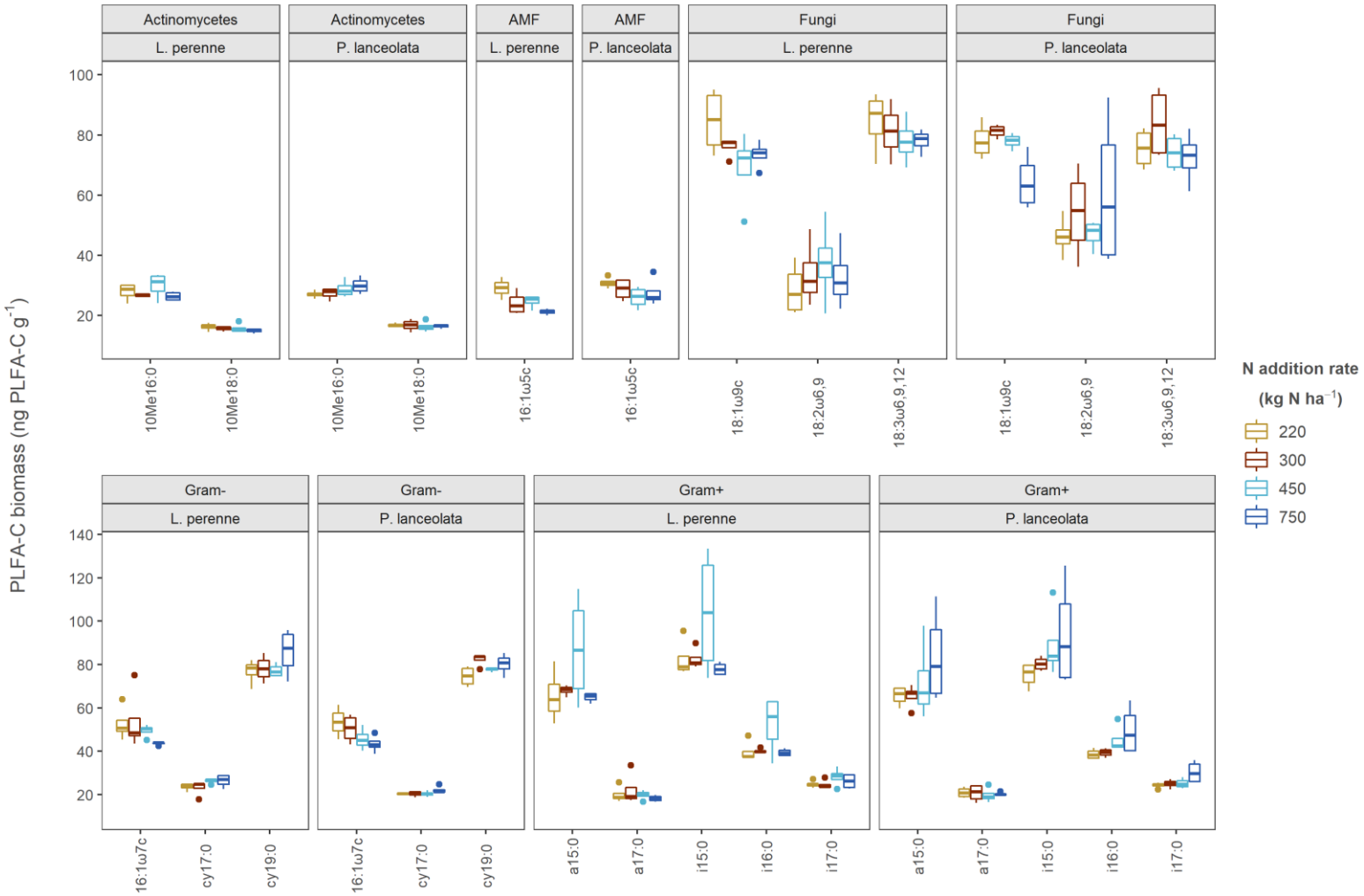

Figure A.2.2. Phospholipid fatty acid-C (PLFA-C) concentrations of individual biomarkers (ng PLFA-C g^{-1}) within microbial groups for each plant species and N treatment (kg N ha^{-1}).

A.3 Supplementary Material for Chapter 5

Table A.3.1. Mean ± standard deviation of plant biomass for each experimental biomass and N addition treatment (see Table 5.1 in the main text for treatment descriptions).

Treatment	**Aboveground biomass (g cm^{-2})**	**Belowground biomass (g cm^{-3})**
$M_fC_1N_0$	267 ± 33	5005 ± 1330
$M_fC_1N_1$	308 ± 58	7280 ± 853
$M_iC_1N_0$	268 ± 54	4640 ± 1112
$M_iC_1N_1$	356 ± 65	5044 ± 4050
$M_iC_0N_0$	235 ± 17	8709 ± 4067
$M_iC_0N_1$	315 ± 35	12683 ± 7351
M_0N_0	2102 ± 226	1877 ± 1267
M_0N_1	2037 ± 323	7367 ± 4673

Table A.3.2. Enzyme substrates and buffers with adjusted pH used for enzyme activity assays. MUB = modified universal buffer.

Enzyme (abbreviation)	**Substrate (concentration)**	**Buffer (pH)**
β-1,4-glucosidase (BG)	*p*-nitrophenyl-β-D-glucopyranoside (25 mM)	MUB (6.0)
β-1,4-N-acetyl-glucosaminidase (NAG)	*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (10 mM)	Acetate buffer, 100 mM (5.5)
Leucine aminopeptidase (LAP)	L-Leucine-*p*-nitroanilide (2 mM)	MUB (7.5)
Acid phosphatase (AP)	*p*-nitrophenyl-phosphate (25 mM)	MUB (6.5)

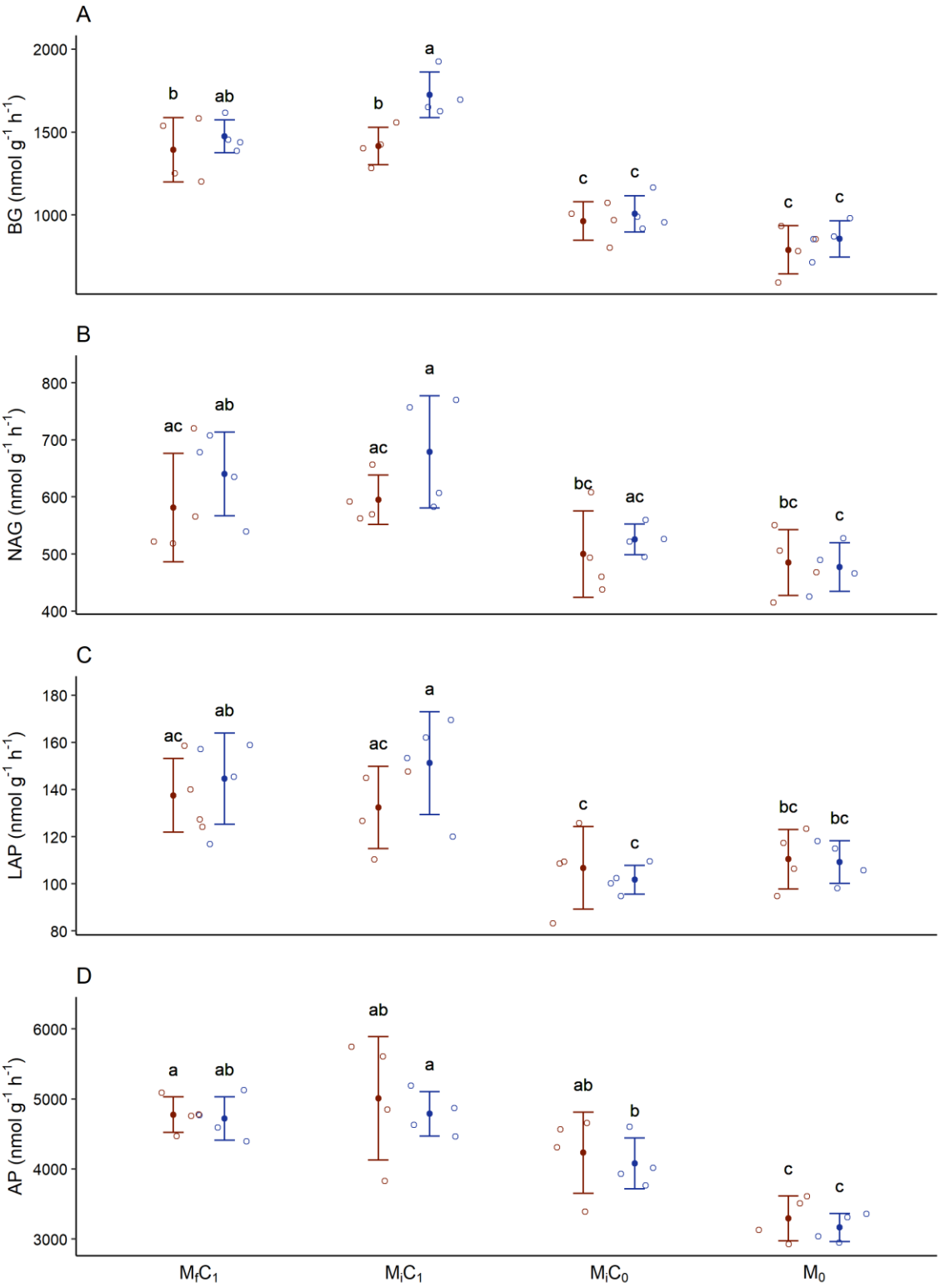

Figure A.3.1. Activities of extracellular enzymes β-1,4-glucosidase (BG) (A), β-1,4-N-acetylglucosaminidase (NAG) (B), leucine aminopeptidase (LAP) (C), and acid phosphatase (AP) (D) in response to biomass management (*x*-axis, for label identifiers see Table 5.1 in the main text) with (blue) and without (red) nitrogen addition. Closed points and error bars represent means and standard deviations, respectively. Observations are scattered randomly as open points around the mean. Different letters indicate significant differences among the biomass and N treatments ($P < 0.05$).

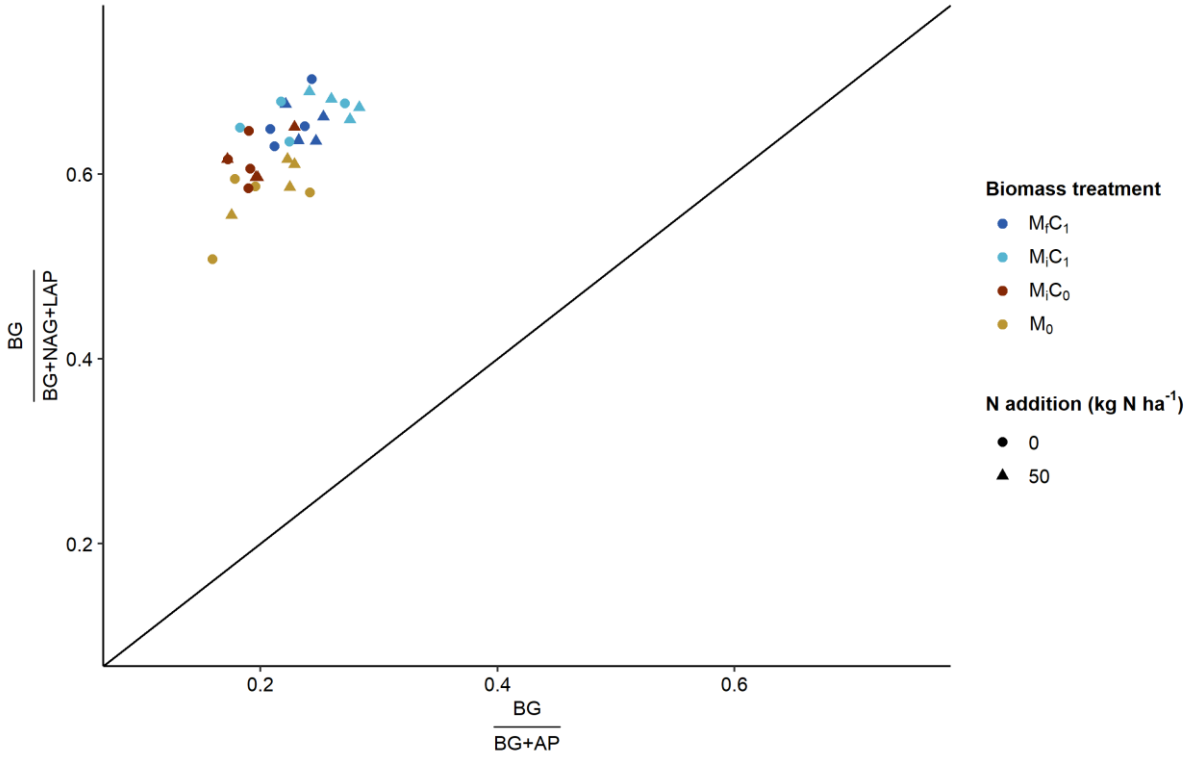

Figure A.3.2. Eco-enzymatic stoichiometry of the relative proportions of C- to N-acquiring versus C- to P-acquiring enzyme activities. β-1,4-glucosidase activity (BG) represents C acquisition, β-1,4-N-acetyl-glucosaminidase (NAG) and leucine aminopeptidase (LAP) represent N acquisition, acid phosphatase (AP) represents P acquisition. Data points above and below the 1:1 line (solid) indicate strong metabolic P and N limitation, respectively. The length of the vector from the plot origin indicates metabolic C limitation.

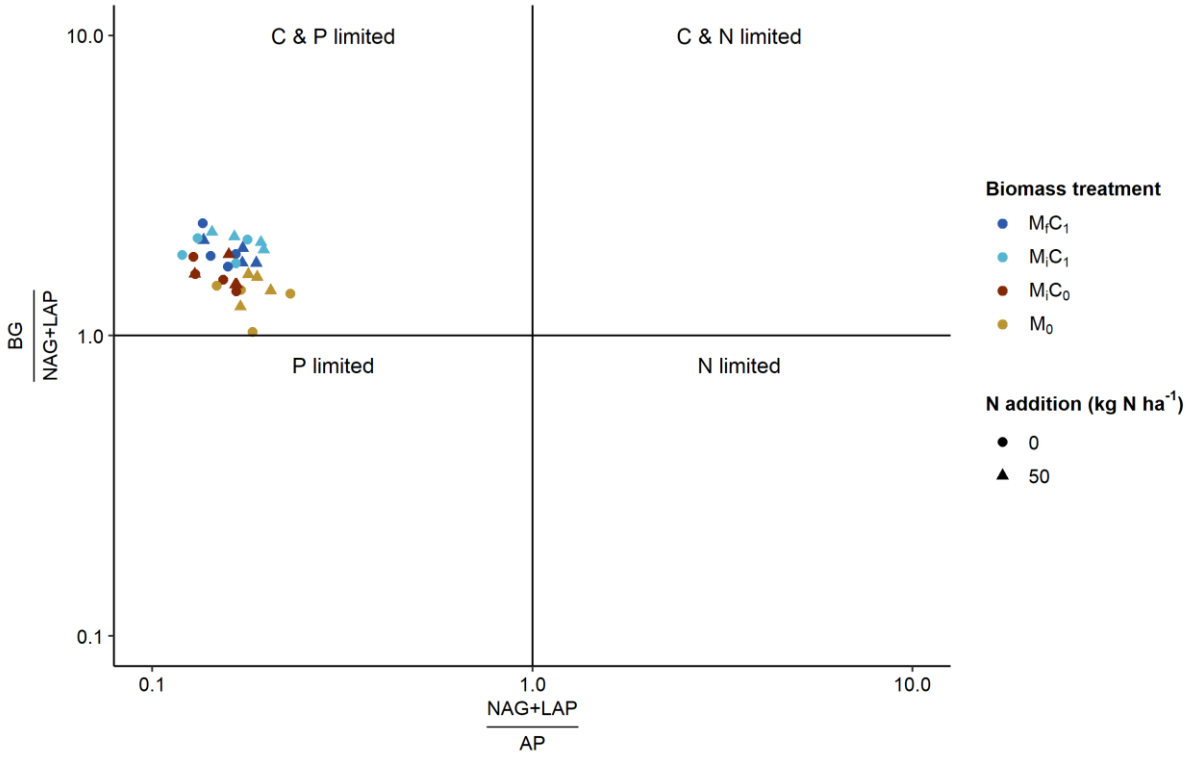

Figure A.3.3. Eco-enzymatic stoichiometry of the ratios of C- to N-acquiring versus N- to P-acquiring enzyme activities indicating patterns of microbial elemental limitations. β-1,4-glucosidase activity (BG) represents C acquisition, β-1,4-N-acetyl-glucosaminidase (NAG) and leucine aminopeptidase (LAP) represent N acquisition, acid phosphatase (AP) represents P acquisition. Solid lines represent thresholds where substrate limitation shifts between elements. Axes were log-transformed.

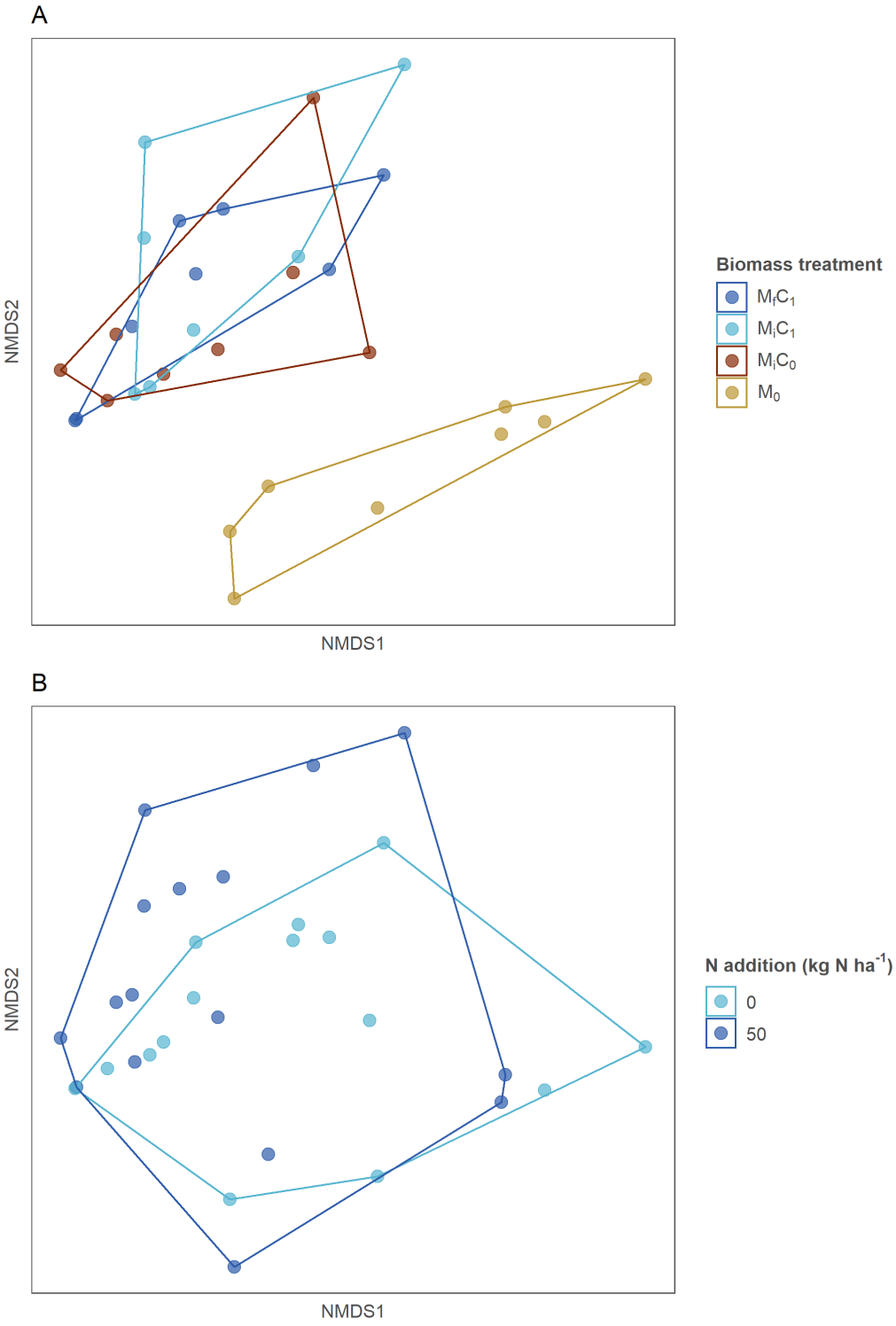

Figure A.3.4. Non-metric multidimensional scaling (NMDS) ordination based on distance matrix calculated from relative PLFA biomarker abundances (mol%) of biomass (A) and N fertilisation (B) treatments.

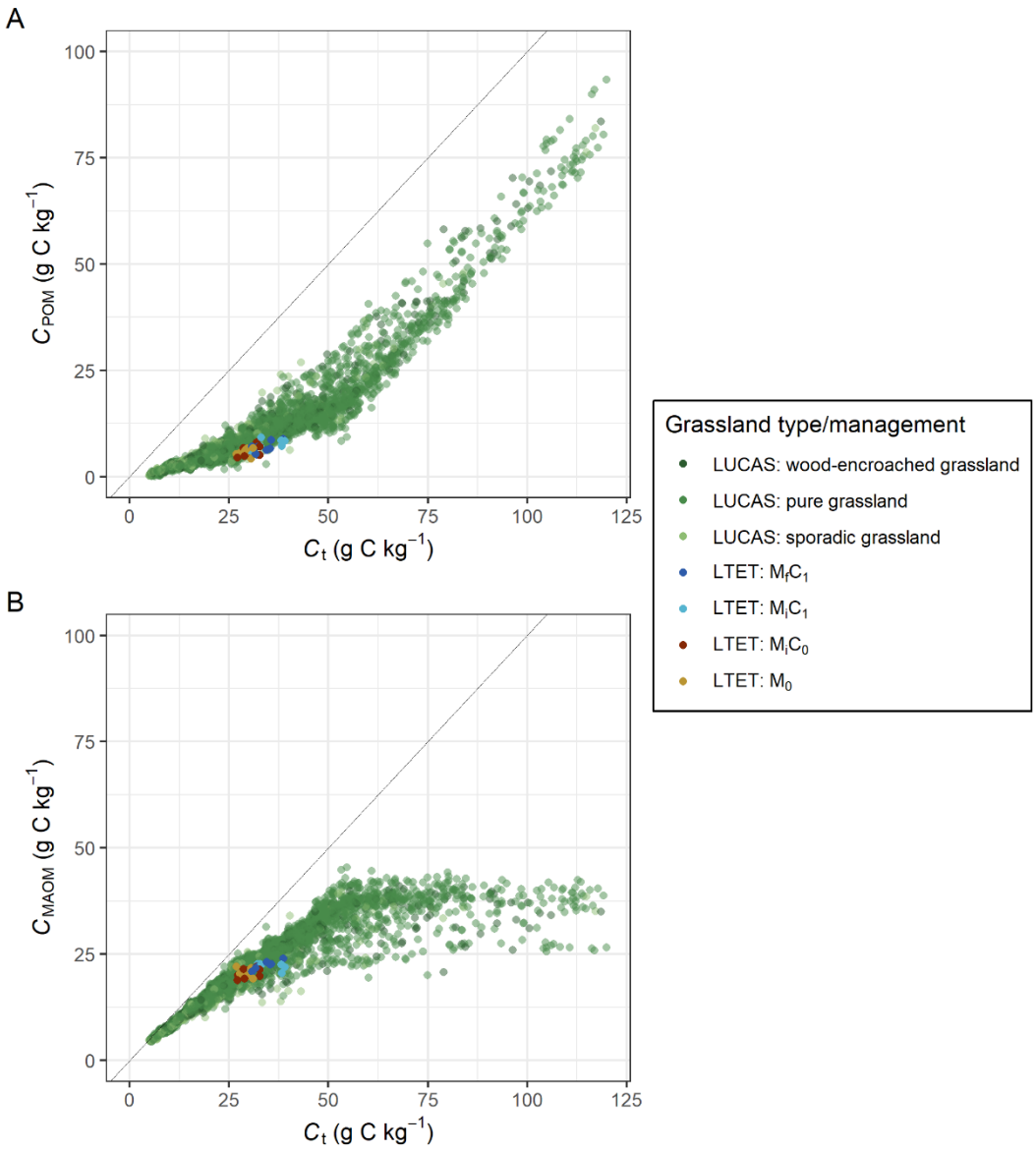

Figure A.3.5. Distribution of soil organic C concentrations in particulate (C_{POM}) (A) and mineral-associated organic matter (C_{MAOM}) (B) fractions versus total soil organic C concentrations (C_t) for the different biomass treatments of the long-term ecology trial (LTET) in comparison to European grassland data from the Land Use/Land Cover Area Frame Survey (LUCAS). 1:1 lines were added for visual aid. LUCAS data were originally published by Cotrufo et al. (2019) and are used here for comparative reasons only.

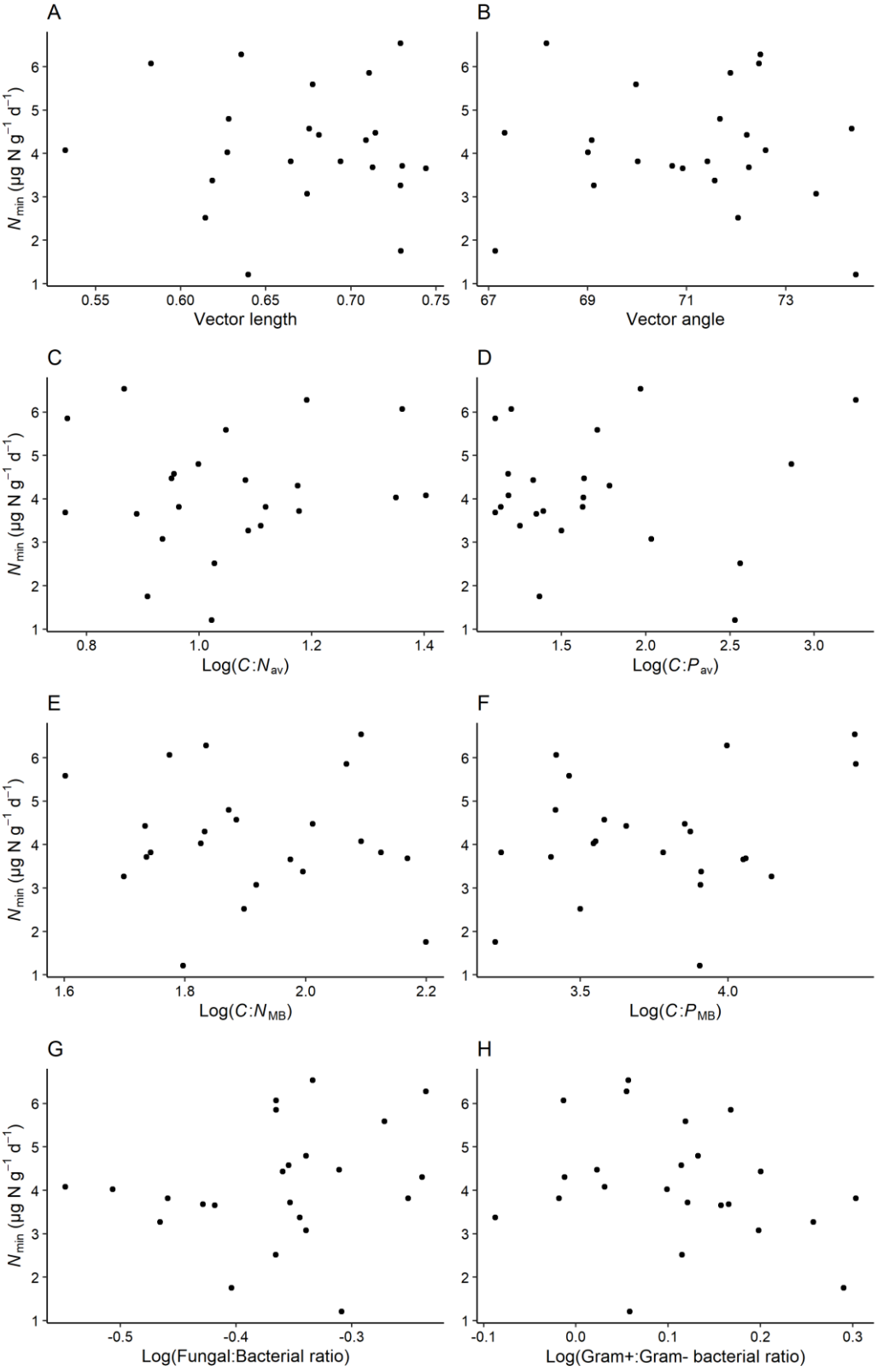

Figure A.3.6. Relationships between gross NH_4^+ mineralisation rate (µg N g^{-1} d^{-1}) and vector length (A) and vector angle (B), available C:N (C:N_{av}) (C) and available C:P (C:P_{av}) (D), microbial biomass C:N (C:N_{MB}) (E) and microbial biomass C:P (C:P_{MB}) (F), fungal:bacterial ratio (G), and gram-positive:gram-negative bacterial ratio (H). No significant relationship was found ($P > 0.05$).

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